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**DEVELOPMENT OF A NOVEL ULTRA-SENSITIVE
IMMUNOASSAY FOR THE DETECTION OF PRION
PROTEIN ON
SURGICAL INSTRUMENTS**

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A thesis submitted for the degree of Doctor of Philosophy

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**Centre for Emergency Preparedness and Response
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**Porton Down
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Abstract

The detection of the infectious agents responsible for prion diseases remains problematic. The unusual nature of this infectious agent means that it is not inactivated by standard decontamination and sterilisation processes and there is evidence of transmission through blood transfusion. There is an urgent requirement for an assay to provide reliable validation of novel decontamination processes and a sensitive test for screening of blood donations and diagnosis of patients.

This research project aimed to address these problems, by the development of a novel ultra-sensitive detection method based on a thermostable adenylate kinase (tAK) antibody label coupled to ATP-bioluminescence. Utilisation of thermostability of AK enabled inclusion of background reducing heat steps within the format therefore enabling detection of specific antigen within a background of proteins.

The thesis describes development of the methodology from initial model assay to identification and production of a suitable recombinant tAK. An attempt to produce sensitive anti-prion polyclonal antiserum is also described. Finally, the use of the monoclonal antibody 6H4 (Prionics) labelled with tAK via a cleavable linker to establish limits of detection of the assay is described and established as 15 pg.mL^{-1} of rec PrP using a fully optimised assay (670 fM). Sensitive detection of rec PrP within relevant biological substrates was also shown, along with some detection of infectious BSE (301V) MBH. There was also some evidence of detection on the surface of surgical grade stainless steel.

The use of this AK-ELISA is discussed within current scientific knowledge and specifically the continuing lack of available sensitive assay. The sensitivity achieved using

this assay format was ultimately not sufficient to address this requirement, however the developed detection methodology has the flexibility to be coupled to new emerging technologies or disease specific antibodies, to provide an immediate increase in sensitivity in answer to this continuing need.

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Abbreviations

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
AK	Adenylate kinase
AK	Myokinase
BCG	Bacille Calmette-Guerin
BSE	Bovine Spongiform Encephalopathy
CJD	Creutzfeldt-Jakob disease
DH	Department of Health
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FSE	Feline Spongiform Encephalopathy
FFI	Fatal Familial Insomnia
FPLC	Fast Protein Liquid Chromatography
GSS	Gerstmann-Straussler-Scheinker disease
HPA	Health Protection Agency
iMBH	mouse passaged BSE (301V) infectious mouse brain homogenate
IPCR	immuno-polymerase chain reaction
KLH	keyhole limpet haemocyanin
L/L	luciferin-luciferase reagent
NCJDSU	National Creutzfeldt-Jakob Disease Surveillance Unit
PBS	Phosphate buffered saline
PBS-T	PBS containing 0.05 % Tween 20

PCR	Polymerase chain reaction
PMCA	Protein misfolding cyclic amplification
PPD	Tuberculin Purified Protein Derivative
PrP ^C	Normal protease sensitive isoform of the cellular prion protein PrP ^C characterised mainly by alpha helices content.
PrP ^{Sc}	Abnormal protease resistant and putative disease associated isoform characterised by large β -sheet content
rec PrP	Recombinant PrP
RLUs	Relative light units
sCJD	Sporadic CJD
SEAC	Spongiform Encephalopathy Advisory Committee
SEM	Scanning electron microscopy
SSDs	Sterile service departments
TB	Tuberculosis
TMB	Tetramethybenzidine
TSEs	Transmissible spongiform encephalopathies
vCJD	Variant Creutzfeldt-Jakob disease

Glossary

archaea	A group of microorganisms which are distinct from bacteria and single celled prokaryotes
bioassay	<i>In vivo</i> assessment of infectivity of prion infectious agent and currently the 'gold standard' test
bioluminescence	Measurable light emission by biological processes found in certain bacterial systems, several marine animals and in fireflies
Iatrogenic CJD	Represents surgical transmission of sCJD and inherited CJD and associated mainly with transplantation of dura mater grafts, ocular tissue and use of human growth hormone derived from pituitary glands of cadavers.
Kuru	An acquired form of prion disease associated with the practice of ritualistic cannibalism which took place in Papua New Guinea and reached epidemic levels in the 1950s
leucodepletion	Removal of white cell component of blood donations
mesophiles	The name for organisms that grow best at temperatures between 15- 40 °C
Prion	Unique infectious agent currently believed to be infectious protein
PrP ^C	Normal protease sensitive isoform of the prion molecule
PrP ^{Sc}	Putative disease associated protease resistant isoform of the prion molecule

Thermophiles

The name for organisms that can survive at temperatures above 45 °C with further categories of hyperthermophiles with optimum temperatures for growth in excess of 80 °C

variant CJD

Identified in the UK in the 1990s linked to a younger age of onset and thought to be associated with consumption of BSE contaminated meat products

1 Literature Review

1.1 Transmissible spongiform encephalopathies (TSEs)

Transmissible spongiform encephalopathies (TSEs) are a family of related neurodegenerative disorders which affect numerous animal species in addition to humans. The animal forms of disease include Bovine Spongiform Encephalopathy (BSE) in cattle, Feline Spongiform Encephalopathy (FSE) in cats, Mink Spongiform Encephalopathy, scrapie in sheep and Chronic Wasting Disease in deer. The human forms of these diseases include Creutzfeldt-Jakob disease (CJD), Kuru, Gerstmann-Straussler-Scheinker disease (GSS) and fatal familial insomnia (FFI) and all these disorders are invariably fatal and associated with a range of clinical and pathological presentations (Collinge 2001; Prusiner 1998; reviewed by Wadsworth & Collinge 2007). The human forms of these diseases have a diverse range of clinical presentation however they all exhibit long incubation periods of months or in some instances years without any symptoms of infection or immune response.

1.1.1 Prions

It has been widely accepted that TSEs are caused by a unique infectious agent, which has no nucleic acid and appears composed only of protein and which has been termed a prion. The prion hypothesis was proposed originally by Stanley Prusiner (Prusiner 1982), but there is still considerable debate surrounding this theory (Chesebro 2003). This group of diseases are associated with the formation of a protease resistant form (PrP^{Sc}) of a normal cellular protein (PrP^{C}), which is a 30-35 kDa glycoprotein found in most cells (Collinge 2001; Prusiner 1998). The normal form of the cellular protein PrP^{C} has a structure mainly

composed of alpha-helices with a lesser beta-pleated sheet content. The abnormal form PrP^{Sc} is characterised by the presence of a large β -sheet content and which results in the aggregation of the abnormal prion protein in the brain leading to spongiform changes (Prusiner 1998).

The prion hypothesis proposes that it is this abnormal protein molecule that is the infectious agent and is able to cause conversion of PrP^C to the abnormal PrP^{Sc}. TSEs are associated with increased resistance of the protein molecule to protease digestion which results in a characteristic banding pattern on SDS-PAGE and Western blotting analysis. The resulting distinctive patterns can be used to differentiate between different strains of disease. The use of Western blot technique confirms immunoreactivity of the material after protease digestion and therefore verifies the presence of the putative disease associated isoform PrP^{Sc}. PrP^C protein is encoded by a single gene and it has been found that a polymorphism at codon 129 for methionine or valine has been associated with differing susceptibility to prion diseases (Bishop *et al.*, 2006). Methionine homozygosity is associated with reduced incubation period of many forms of CJD in general and very specifically with variant CJD (vCJD). These diseases are relatively rare and affect approximately one person per million of the population and the human forms of the disease can be categorised as sporadic, inherited, or acquired in presentation (Prusiner 1998).

1.1.2 Sporadic CJD

Sporadic CJD (sCJD) is the most common of the human forms and approximately 85% of cases fall into this category with an occurrence rate at about 1-2 cases per million of the population (reviewed by Wadsworth & Collinge 2007). The onset of the sporadic form of the disease is generally between the ages of 45-75 with the median age of death occurring

at 68 years and the clinical presentation tends to be associated with a rapid progression and short duration of ~5 months, although atypical forms of the disease with longer duration of illness have been recognised. The origins of this form of CJD are unclear and no specific mutation has been associated with it (Prusiner 1997) and there are a number of different hypotheses. Spontaneous mutation of normal PrP^C to the abnormal isoform PrP^{Sc} has been suggested (Prusiner 1989). Other theories have centred round the possibility of horizontal transmission of infection from human or animal sources (Gajdusek 1977), however no convincing evidence for this has been found.

1.1.3 *Inherited CJD*

Inherited CJD is the cause of approximately 15% of cases of CJD in humans and is associated with a known genetic mutation in the PRNP gene. Although the exact nature of the pathogenesis is currently unknown, it is thought to be caused by an increased tendency of the normal protein PrP^C to convert to the abnormal PrP^{Sc} form (Brown *et al.*, 1987). This has not been completely accepted (Wadsworth & Collinge, 2007). The inherited diseases GSS, FFI and CJD have different clinical features and duration of illness.

1.1.4 *Acquired CJD*

Acquired human CJD includes Kuru, variant CJD (vCJD) and iatrogenic CJD. Kuru is an acquired form of prion disease associated with the practice of ritualistic cannibalism which took place in Papua New Guinea. The disease had reached epidemic levels in the 1950s and 60s before the practice of cannibalism ended. Epidemiological analysis of the progression of this disease both during and post epidemic has revealed incubation periods of up to 50 years and this has raised the possibility of potentially long asymptomatic

phases in the case of other prion diseases such as vCJD (Collinge *et al.*, 2006; Wadsworth & Collinge, 2007).

Another example of an acquired CJD is iatrogenic CJD, which occurs in cases of disease transmission resulting from medical treatment (Brown, Preece & Will, 1992). Iatrogenic CJD is not a distinct strain as such but represents surgical transmission of sCJD and inherited CJD. Transmission of sporadic CJD has been reported due to transplantation of ocular tissue (Tullo *et al.*, 2006) and also associated with transplantation of dura mater grafts (Lang *et al.*, 1995). Surveillance studies in the UK identified seven cases of iatrogenic CJD associated with dura mater grafts in the period between 1970-2003 (Heath *et al.*, 2006). Iatrogenic CJD has also been reported as a result of the use of human growth hormone which was derived from cadavers (Brown, 1998). A review of iatrogenic cases of CJD (Brown *et al.*, 2006) notes that cases are declining now due to changes in practice following the discovery of this potential route of infection, although there are still occasional occurrences which are perhaps due to extended incubation periods.

1.1.5 Variant CJD

A variant form of CJD was identified in the UK in the 1990s linked to a younger age of onset and is thought to be associated with consumption of BSE contaminated meat products. This was subsequently shown to be derived from the same strain on experimental analysis (Bruce *et al.*, 1997; Collinge *et al.*, 1996). This disease differed from sporadic and inherited CJD in its clinical presentation and young median age of onset of ~26 years. The appearance of this new strain of human disease has raised widespread concerns of a potential public health threat. In addition, the long incubation periods and unusual stability of the infectious agent, which is resistant to the usual methods of decontamination and

sterilisation, has raised the possibility that a proportion of the population could potentially be harbouring this disease unknowingly and could result in iatrogenic presentations through surgery, blood donation and organ transplantation etc. An additional feature specific to vCJD is the presence of lymphoreticular accumulation of disease associated PrP^{Sc} in spleen, tonsil, appendix and lymph nodes. This is different to other forms of CJD in which disease associated PrP^{Sc} accumulation has been demonstrated only in the central nervous system (Hilton *et al.*, 2004). This may potentially increase the possibility of iatrogenic transmission of vCJD via tissues such as tonsil and spleen.

Initially there were widespread fears that this disease was going to reach epidemic levels, however to date, the total number of deaths from definite or probable vCJD is 163 (March, 2008) with a further 3 cases incubating disease (The National Creutzfeldt-Jakob Disease Surveillance Unit (NCJDSU) 2008) which in terms of numbers affected this is relatively low and the numbers appear to be declining after a peak around 2002-3. This has resulted in speculation that the vCJD “epidemic” is coming to an end and therefore this disease no longer poses a risk to public health. However some recent retrospective prevalence studies looking at archived pathological tonsil and appendix specimens have raised the possibility that there a number of people with sub clinical manifestations incubating the disease unknowing and that the prevalence in the population may be higher than suspected (Hilton *et al.*, 2004; Ironside *et al.*, 2006). This combined with growing understanding of the exceptionally long incubation periods through analysis of Kuru (Collinge *et al.*, 2006) has meant that there is still concern over the possible public health issues associated with vCJD.

1.2 Decontamination of surgical instruments – the issues

Due to the unique properties of this infectious agent a number of questions need to be answered urgently to address public health concerns and therefore should be the focus of research (Ironside, 2006). These include the development of a screening assay which is specific, sensitive and flexible to enable detection in blood, the development of an effective treatment and the development of a validated decontamination method for processing of surgical instruments.

The remarkable resistance of the prion infectious agent to standard methods of decontamination and cleaning has been highlighted (Taylor, 1991; reviewed by Taylor, 2000). A number of standard physical and chemical methods of decontamination were assessed in these studies, including dry heat; autoclaving; acids and bases; alkylating agents, detergent solvents and proteolytic enzymes. The results were startling as it was revealed that a number of the methods such as use of alcohols, aldehydes, and autoclaving did not eliminate infectivity, and far from inactivating the infectious agent, some made it more stable. It was also apparent that the most thermostable of all the strains tested was BSE 301. (Taylor, 1991; reviewed Taylor, 2000; Fernie *et al.*, 2007). As a result of these studies, it was concluded that a combination of methods that aimed to disrupt the protein structure would be more effective and that BSE 301V would be the best model for testing any decontamination regimes to enable assessment of the worst case scenario.

The resistance of the infectious agent to standard methods of decontamination has been further substantiated by reports of iatrogenic transmission resulting from inadequate decontamination of neurosurgical instruments. One report details the results of a study using an instrument which had been used on a known case of CJD, and treated with 70% alcohol and formaldehyde vapour for sterilisation prior to storage for two years and

thereafter implanted into a chimpanzee that later developed disease (Bernoulli *et al.*, 1977). Although the methods by which the instruments were sterilised would not currently be considered adequate, the experiments raised concerns over the safety of neurosurgical instruments, especially given the long time span which had elapsed before transmission of infectivity. Cases have also been associated with neurosurgical / electroencephalography (EEG) electrode contamination (Will & Matthews, 1982) and a further five cases of possible iatrogenic transmission have been identified subsequently (reviewed by Brown *et al.*, 2000).

A epidemiological case control study (Collins *et al.*, 1999) was conducted comparing a group of patients with known or suspected CJD and a control group from the normal population which found that there was an increased association of at least 3 episodes of surgery with CJD and it has been postulated that this could be due to contamination present on surgical instruments. Furthermore, the authors found that the risk associated with surgical procedures increased over time and multiple operations.

In response to epidemiological studies and reports of iatrogenic transmission via contaminated surgical instruments combined with increasing knowledge regarding the resistance of the infectious agent to standard methods of decontamination and sterilisation, has resulted in risk assessments being undertaken by the Department of Health (DH). This has lead to the introduction of measures designed to reduce this potential risk to public health, including the incineration of all instruments used in surgery on known cases of CJD and quarantining of 'at risk' cases. Reports of transmission of vCJD by blood and blood products (Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006) has led to greater numbers of cases being considered within this 'at risk' category including haemophiliac patients who were recipients of clotting factors and this has led to increasing numbers of surgical instruments being quarantined, including high cost instruments such as

endoscopes. There have been vastly different estimates of the prevalence of infection in the UK population. One retrospective study (Hilton *et al.*, 2004) tried to estimate the prevalence of vCJD by the use of immunohistochemistry to examine an archive of appendix and tonsil pathology samples. The results were somewhat surprising as 3 appendix samples showed an accumulation of the abnormal prion protein indicative of vCJD out of ~12,000 samples. This suggested that the prevalence of vCJD may be much higher than previously believed at around 237 persons per million of the population. This result highlights that the levels of risk are still unknown and may be higher than suspected and provides further support of the precautionary approach taken by the DH to reduce risk to the public.

There is clearly an urgent requirement for a reliable method of decontaminating surgical instruments. Treatments known to offer a significant log reduction in infectivity such as sodium hypochlorite (20,000 ppm active chlorine) would not be a practical method for routine use due to safety concerns for operators and potential damaging effect on surgical instruments. Alcohols have a well known bactericidal effect and they are often used in conjunction with formaldehyde for the cleaning and chemical sterilisation of certain surgical instruments including neurosurgical instruments such as electrodes. However, one study reports the potential for alcohols to fix prion proteins more tightly to the surface of surgical steel and recommends that alcohols should not be used for sterilisation of neurosurgical instruments (Prior *et al.*, 2004).

As a result of concerns over the potential transmission of CJD via contaminated surgical instruments, questions arose regarding the efficacy generally of cleaning and decontamination processes currently taking place in sterile service departments (SSDs) across the NHS. In order to get an idea of the baseline standard, the DH commissioned an anonymised study of actual used and reprocessed instruments from different hospitals,

covering different types of surgical procedures and collected at the point of return to use. The results of these studies (Baxter *et al.*, 2006; Lipscomb *et al.*, 2006; Murdoch *et al.*, 2006) revealed that levels of residual protein on the surface of surgical instruments after processing typically ranged from 200 µg to over 2 mg. Indeed one instrument examined had a very high protein load of 45 mg. In common across the studies, the highest residual protein levels were associated with the surgical instrument sets used for tonsillectomies.

These findings raised concerns as they demonstrated that a proportion of instruments in normal circulation have significant levels of residual protein contamination. The studies although small, demonstrated variation in general standards of reprocessing of surgical instrument across the UK and that patients could potentially be exposed to significant levels of residual protein from surgical instruments, despite being deemed sterilised. Given the concerns about iatrogenic transmission of historical forms of CJD through contaminated surgical instruments, this could potentially pose a theoretical source for ongoing vCJD transmission. Furthermore the fact that the tonsillectomy instruments were shown to have the highest levels of residual protein was of concern as tonsil tissue has been identified as being a high risk tissue for vCJD with levels of infectivity at only 100 or 1000 fold less than brain material (Bruce *et al.*, 2001).

A question which has been posed is the effect of drying of proteinaceous material on the ultimate efficacy of cleaning methods with obvious implications for iatrogenic transmission of prion infectivity. Concerns over the drying on of biological material on route to Sterile Service Departments (SSDs), has prompted studies. One study (Lipscomb *et al.*, 2007) examined the effects of pre-soaking versus drying on surface protein contamination and found that there was significantly less overall protein contamination levels when pre-soaking was used.

Dental instruments may pose a problem due to their contact with potentially contaminated peripheral nerve tissue. The level of gross contamination on the surface of endodontic files following use and standard reprocessing has been highlighted as a particular problem (Smith *et al.*, 2002; Walker *et al.*, 2007). An assessment of this risk based on existing scientific knowledge and gaps in current understanding led the Spongiform Encephalopathy Advisory Committee (SEAC) to issue a statement that endodontic files and reamers should be single use. <http://www.seac.gov.uk/statements/statement0506.htm>

1.2.1 Novel decontamination methods

There has been a focus by researchers on the development of reliable, validated methods of surgical instrument decontamination. Indeed, there have been a wide range of technologies developed to date and some commercially available products, Hamo 100 (Steris), Prionzyme – M (Genencor) have been independently certified for use (CE marking). Hamo 100 is an alkaline based detergent system, and Prionzyme M is based on proteases within an alkaline base and both have shown relatively good results for removal of prion infectivity (Fichet *et al.*, 2004; Fichet *et al.*, 2007b; McLeod *et al.*, 2004). The action of these products is certainly less harsh than treatment with 1 Normal (1N) solution of sodium hydroxide, however there are still limitations and Hamo 100 is not recommended for use with flexible endoscopes. It is clear that although that these commercially available methods offer reduction in prion infectivity that there is still a requirement for a method of accurately validating the process to show that the required reduction in infectivity is being achieved.

In addition, a number of other decontamination methodologies currently being developed are showing promise (Table 1-1). One group of researchers have reported significant

reduction in RML scrapie infectivity on surgical steel wires as assessed by bioassay, when using a combination of ionic detergents and proteases combined with autoclaving (Jackson *et al.*, 2005). This result is promising, however there are studies showing that there is a difference in the stabilities of scrapie to vCJD and also mouse passaged BSE 301V (reviewed by Taylor, 2000) and it is therefore difficult to accurately gauge and compare methodologies (reviewed by Sutton *et al.*, 2006).

Bioassay is currently the most reliable method of accurately and sensitively assessing levels of infectivity and therefore is considered the “gold standard” and therefore all current decontamination methodologies are required to prove their efficacy *in vivo* using a suitable animal model. Bioassay methods vary markedly from the infectious agent utilised e.g. scrapie and mouse passaged BSE 301V, and the species of animals challenged e.g. mouse and hamster. In addition the various methods of delivery of the infectious agent are currently reported ranging from the use of stainless steel wires inoculated intra-cerebrally to stainless steel spheres, which aim to mimic exposure via surgical instruments (Ferne *et al.*, 2007). However, the most reliable and measurable way of assessing the reduction in infectivity by decontamination methodologies is by direct intra-cerebral inoculation of potentially infectious material and assessing the survival data of the animal model used (reviewed by Sutton *et al.*, 2006).

The efficacy of gaseous hydrogen peroxide has also been assessed for reduction of infectivity of the hamster 263K scrapie strain and the results showed some success both *in vitro* and *in vivo*, demonstrating >4.5 log reduction (Fichet *et al.*, 2007a). One main target of the development of new technologies is the challenge to develop practical methods easily transferable to current NHS sterile SSDs. Therefore this type of technology has the benefit that it is an already ‘tried and tested’ method within industry and used successfully on a wide range of surfaces however this log reduction in infectivity is not sufficient to

render surgical instruments safe in terms of decontamination. Another study reports on efficacy of a method normally used for the pasteurisation of food (Heindl *et al.*, in press). This methodology has shown significant reduction in 263K scrapie in the region of 6-8 logs, however again it is difficult to accurately compare methodologies.

The use of gas plasma has also been assessed for decontamination of prion protein on the surface of surgical instruments and again with the advantage that it is also a recognised methodology within industrial settings. This technology has the proven suitability for cleaning and sterilisation of a range of materials and surfaces, thereby showing potential for application across the wider range of surgical instruments for processing. One report has been published (Whittaker *et al.*, 2004) on the efficacy of gas plasma for cleaning dental endodontic files, and which was evaluated by visual assessment of the levels of surface contamination by scanning electron microscopy (SEM) before and after plasma cleaning. The results indicated that all organic matter had been fully removed by the cleaning process with no obvious damage to the instrument surface. A further study (Baxter *et al.*, 2005) examined the application of this technology in comparison to stringent washing procedures, for the removal of 263K scrapie prion infectivity from the surface of stainless steel spheres and surgical instruments. The results of SEM analysis followed by bioassay showed complete removal of proteinaceous material and infectivity. The authors make the point that in the absence of a validated method of removal of prion infectivity that the establishment of a method to ensure the complete removal of protein residue from the instrument surface is of particular importance.

Infectious Agent	Animal	Method	Log₁₀ Reduction of infectivity	Reference
263K scrapie strain	hamster	Hamo-100	>4.5	Fichet <i>et al.</i> , 2007a
263K scrapie strain	hamster	Gaseous hydrogen peroxide	>5.5	Fichet <i>et al.</i> , 2007b
263K scrapie strain	hamster	Pasteurisation	6-8	Heindl <i>et al.</i> , in press
BSE 301V (mouse passaged)	mouse	Prionzyme-M (Genencor)	>4	McLeod <i>et al.</i> , 2004
263K scrapie strain	hamster	Gas plasma	Removal of infectivity beyond detection	Baxter <i>et al.</i> , 2005
RML scrapie	hamster	Combination of ionic detergents and proteases	Not reported	Jackson <i>et al.</i> , 2005

Table 1-1 Comparison of reduction of infectivity by decontamination methodologies

A number of initiatives to improve the standards of cleaning and sterilisation generally within the NHS has been started and there is clearly a great deal of research taking place focussed on the development of specific and practical methodologies to inactivate TSEs, and ultimately to provide reliable processes for use in SSDs to ensure the safety of processed surgical instruments with respect to CJD.

For any of these novel technologies summarised in Table 1-1, to be taken forward for use in SSDs for decontamination of high risk instruments as well as relatively low risk instruments there has to be a reliable method of assessing and validating methods before implementation.

One of the difficulties is the requirement of the use of bioassays as the only reliable way to measure whether prion infectivity has been reduced or removed. The development of a reliable and consistent method for conducting a bioassay has been a considerable challenge; however the importance of the establishment of a benchmark to enable comparison of new technologies cannot be underestimated. It has been raised however,

that assessment should utilise a relevant TSE strain, in order to ensure confidence in a methodology or product (Sutton *et al.*, 2006). This is particularly important when a number of developed methodologies are showing considerable promise for transfer into practical use within the near future. In addition to validation of the efficacy of a novel methodology at a developmental stage, there is an urgent requirement for the provision of a process indicator to provide quality assurance of the reliability of the process once in place within SSDs and therefore this is also a requirement to enable integration of novel methods.

1.2.2 *Blood and blood products*

The possible public health concerns associated with vCJD were further strengthened with the publication of a report of possible transmission through a blood transfusion. (Llewelyn *et al.*, 2004) The recipient in this case had received blood from a donor who had developed vCJD 3 years after donating. The recipient subsequently developed vCJD > 6 years after receiving the transfusion. There is no epidemiological evidence to support transmission of other forms of CJD, such as sporadic CJD, via blood transfusion (reviewed by Ludlam & Turner, 2006), but the publication of this article raised the possibility that this might not be the same for vCJD. The initial case was identified following establishment of a UK surveillance programme to assess the transmissibility of CJD by blood and blood products. A second probable case was identified in which the patient died of other causes but was known to be the recipient of blood from a donor who subsequently developed CJD. On post mortem, evidence of prion infectivity was found in the spleen and a lymph node and further analysis revealed that this patient was heterozygous at codon 129 (Peden *et al.*, 2004).

In response to growing concerns for the safety of the national blood stocks a number of measures were implemented nationally to lessen the risk. These include the use of surveillance and tracing of blood donations; sourcing of plasma for production of blood components from outside the UK; leucodepletion of all blood used for transfusion and exclusion from the donor pool of individuals who have received a transfusion in the UK since 1980.

There are a small number of individuals within the UK, who have been known to have received blood transfusions from donors who subsequently developed vCJD. A third case within this group was reported (Wroe *et al.*, 2006) and subsequently a fourth case was announced via a press statement by the Health Protection Agency (2007).

(http://www.hpa.org.uk/webw/HPAweb&HPAwebStandard/HPAweb_C/1195733711457?p=1171991026241) It has been reported that there are a further 23 people within this defined group, who have not as yet been diagnosed with vCJD.

(http://www.hpa.org.uk/hpa/news/articles/press_releases/2007/070118_vCJD.htm)

The measures and restrictions which have been put into place to reduce the risk from blood transfusion in the UK have impacted on the national supplies of blood and blood products. The development of a suitable screening test has therefore become a goal for researchers within this field along with estimation of sensitivity required to enable an assay to reliably detect infectivity in blood for the purposes of ante mortem diagnosis and screening of blood stocks. To date there is still a lack of an assay with sufficient sensitivity to meet this challenge (reviewed by Grassi *et al.*, 2008).

There is still some debate regarding the actual levels of infectivity present in blood and blood products. A review article (Brown, 2005) collected data from a large number of

infectivity studies in which surprisingly the *in vivo* models show a similar picture for sCJD as vCJD in blood, which raises questions about the assumed safety of blood from sCJD individuals. The infectivity in blood is mainly associated within the leucocyte fraction. The exact nature of the infectivity and circulating levels are currently unknown and rely largely on animal studies particularly rodents. This is the only animal model in which blood has been titrated in order to determine levels of infectivity and the results show that the levels are many logs lower than in the brains of the infected animals (reviewed Brown, 2005). The assumption is that the levels of PrP^{Sc} in human blood would also be very low, and therefore sufficient sensitivity would be required to detect several logs less than post mortem tests on brain in order for a blood assay to be useful. One study, Brown (2001), estimated that PrP^{Sc} could be expected to be present at levels of 100 infectious units per mL of buffy coat, which is equivalent to 1-10 pg.mL⁻¹ and other estimates have been set 10-fold lower at 10 infectious units per mL of peripheral blood (equivalent to 0.1-1 pg.mL⁻¹) (Cervenakova *et al.*, 2003). In addition, there are other problems associated with detection of TSEs in blood. Most of the assays currently being developed rely on spiking of either recombinant PrP or infectious brain material from animal studies within blood or sera as there is shortage of blood samples from CJD patients due to ethical reasons. However there is insufficient scientific evidence on whether the form of the infectious molecule is the same in circulating blood as within the brain and indeed it has been postulated that this is unlikely. This may mean that assays developed in this way may not be reliable (Brown, 2005). There is a significant gap in knowledge and understanding regarding prion infectivity within blood despite the obvious urgent requirement for development of a blood assay.

1.2.3 Assays and detection

One of the biggest challenges in the development of a sensitive and specific diagnostic test for CJD is the nature of the infectious agent itself. With no discovered nucleic acid, the use of traditional polymerase chain reaction (PCR) based assays are ruled out and this is combined with a lack of host immune response enabling serology to be used. However there is undoubtedly an urgent requirement for reliable ante-mortem diagnostics, a blood test and method of validating surgical instrument decontamination.

The problem of lack of sensitivity of assays has been a re-occurring theme and for ELISA type formats, this has been mainly due to a lack of specific and sensitive antibodies. A number of different approaches have been used in an attempt to overcome sensitivity issues and indeed prompted the development of the novel ultra-sensitive assay described in this thesis. A number of currently published methods of detection, along with limits of detection achieved have been summarised in Table 1-2.

The use of luminescence has been previously reported as a method of increasing sensitivity and its use in a capture ELISA format for recombinant PrP and bovine brain homogenates from known positive BSE cases, has been described (Biffiger *et al.*, 2002). This assay used 6H4 (Prionics) as a capture system for proteinase K pre-treated brain homogenate and an in-house produced monoclonal antibody conjugate with a chemi-luminescent reporter for detection. The reported limit of sensitivity for this assay was 30 pg.mL⁻¹ of recPrP and BSE positive bovine brain tissue was successfully detected in a blind trial,

Safar *et al* (2002) have developed a conformation dependant immunoassay, designed to detect denatured and native forms of the prion protein utilising the difference in signal between both forms to determine if the sample is positive for disease specific protein and

results showed levels of detection in region of 1ng.mL^{-1} of PrP. Another publication by the same authors (Safar *et al.*, 2005) reports sensitive detection of human prion disease in brain homogenates which exceeded the sensitivity achieved by immunohistochemistry in the same samples. The authors postulate that this is due to detection of sub clinical forms of the disease.

A novel format for detection of PrP^{Sc} has been described which utilises a property of selective binding of PrP^{Sc} to a polymeric ligand. This enables the subsequent washing off of the normal cellular form PrP^C before detection using an ELISA format and this format is currently being developed for commercial use (Microsens) (Lane *et al.*, 2003).

The lack of known nucleic acid sequence in the infectious prion molecule has prevented the use of PCR methodology. However, one research group have developed a very inventive method of cyclic amplification of the protein misfolding of normal cellular protein PrP^C into abnormal disease associated PrP^{Sc} (Saborio, Permanne & Soto, 2001). This process is based on the disruption of aggregates of PrP^{Sc} as it is formed which provides more template for the conversion of PrP^C and has been named protein misfolding cyclic amplification (PMCA). Recently a report of the use of this assay for detection of reduction of scrapie prion infectivity as a result of heating and autoclaving was published (Murayama *et al.*, 2007). Results using this assay were reportedly as sensitive as the concurrent bioassay, thereby raising the possibility of the use of this technique for validation purposes. A version of this approach has been used to detect PrP^{Sc} in blood (Pan *et al.*, 2007). This assay format has also been used to detect prion infectivity in the plasma of GSS infected mice and the assay was shown to detect a sample which had 10 infectious units per mL (Soto *et al.*, 2005), which is approaching the levels of sensitivity required for a human blood test. The use of a method of immunocapillary electrophoresis has also been

evaluated for detection of abnormal prion protein in blood samples of CJD patients, (Lourenco *et al.*, 2006) however the results demonstrated a low sensitivity of ~50%.

Detection of abnormal prion protein in other body fluids such as urine has been attempted. This would have obvious advantages diagnostically in elucidating the pathology of the human prion diseases and clinical risks associated (Head *et al.*, 2005). This study, rather reassuringly, failed to detect the presence of PrP^{Sc} in any of the urine samples from cases of sporadic, variant or familial CJD.

Immunoassays have not been the only approach for detection of CJD on the surface of surgical instruments. The use of microscopic techniques combined with fluorescent reagent have been carried out with detection within the picogram range achieved (Lipscomb *et al.*, 2006). Although it is perhaps unlikely that this methodology could be transferable for routine use in SSDs, it may provide a method for researchers to validate decontamination techniques without the use of bioassay.

The general target of antibody based detection system is the putative disease associated isoform PrP^{Sc}; however this has not been conclusively proved as the TSE infectious agent. One published study shows a strong correlation with 263K hamster scrapie and infectivity (Beekes, Baldauf & Diringer, 1996). However, other studies have failed to find significant accumulation of PrP^{Sc} in infected brain material (Barron *et al.*, 2001; Manson *et al.*, 1999). In addition, there have been a number of reports from experimental studies which show a lack of correlation of infectivity on bioassay with removal of immunoreactivity on Western blots (Lourenco *et al.*, 2006; McLeod *et al.*, 2004). Furthermore, a recently published study (Barron *et al.*, 2007) shows convincing evidence that high titre infectivity can be transmitted from the brains of animals that have shown little or no presence of immunoreactivity for PrP^{Sc}. This therefore demonstrates that the presence of PrP^{Sc} may not

be entirely reliable for the purpose of diagnostics or validation of decontamination and reinforces the importance of bioassays until the nature of the infectious agent itself can be fully understood. However, in the absence of an alternative, this surrogate marker is still commonly used as a marker for infectivity for development of detection assays.

There has evidently been much progress towards the development of sensitive detection assays for CJD for the purpose of validation of decontamination processes and for use in post mortem and blood assays (summarised in Table 1-2). However it is clear that there are still a number of unresolved issues, which have impeded progress of the development of suitable methodologies and thus there is still currently a requirement for validation by bioassay.

Antigen	Method	Detection limit	Reference
Rec PrP	Luminescent ELISA	30 pg.mL ⁻¹	Biffiger <i>et al</i> , 2002
Disease specific prion	Conformation dependant immunoassay	1 ng.mL ⁻¹	Safar <i>et al</i> , 2002
Scrapie	PMCA	10 infectious units per mL	Saborio, Permanne & Soto 2001 Soto <i>et al.</i> , 2005
Rec PrP	Immunocapillary electrophoresis	1 ng.mL ⁻¹	Lourenco <i>et al.</i> , 2006
Rec PrP	ELISA	20-60 µg. mL ⁻¹	Yamamoto <i>et al</i> (in press)
Rec PrP Scrapie	IPCR	1 fg.mL ⁻¹ 10 infectious units per mL	Barletta <i>et al</i> , 2005

Table 1-2 Comparison of limits of detection by published studies

1.3 Ultra-sensitive detection and bioluminescence

The terminology of chemiluminescence and bioluminescence cover the production of measurable light emission by either a chemical reaction or biological processes. Bioluminescence is a relatively rare process in nature, found in certain bacterial systems, several marine animals and in fireflies. In the latter case the enzyme responsible for light production is called luciferase and uses luciferin as a substrate which it converts to oxyluciferin + AMP + light, in the presence of ATP (reviewed by Kricka, 1995).



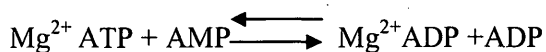
There have been numerous uses identified for this reaction, particularly within biotechnology and products have been developed to enable this reaction to be utilised in a predictable and reliable way including luminometers, reagents and kits. The developed methodologies include the use of reporter gene based assays, biosensors, and also use of ATP released from bacteria upon lysis linked to production of measurable light. The use of ATP linked bioluminescence has been extensively used for hygiene monitoring within the food industry with light emission measured by hand held portable luminometers (Stanley, 1989). However detection levels achieved tend to be quite low and typically in the realms of 10^4 cells with the other drawback that the methodology is non-specific and not able to differentiate between different bacteria particularly the presence of potentially pathogenic species. This technology has been further refined to enable specific detection of bacteria using phage mediated release of adenylate kinase (AK) (Blasco *et al.*, 1998). This method applied the use of specific bacteriophages to lyse cells if present, which was followed by the release of the cell contents which included AK which catalyses the equilibrium reaction:

ATP + AMP \leftrightarrow 2ADP (in the presence of Mg²⁺)

If an excess of ADP substrate is present then the reaction can be driven in the direction of production of ATP, which is the opposite of the cellular metabolism reaction, resulting in amplification of ATP which can be coupled to detection by bioluminescence and measurable light emission. In addition to the possibility of specific detection using AK as marker, an increase in sensitivity of a least 10-100 fold is demonstrable compared to detection of ATP alone. This use of this technology was further described (Squirrell, Price & Murphy, 2002) for specific detection of *Escherichia coli* (*E. coli*) O157. Furthermore it is reported that 40 times as much ATP can be generated within one minute in the presence of an excess of ADP substrate than the initial starting amount (Chittock *et al.*, 1998; Squirrell & Murphy, 1997). This amplification reaction is known to be linear and therefore high sensitivity can be achieved by increasing incubation time, results show detection of 1 cell within an assay time of approximately 25 minutes.

1.4 Thermophilic adenylate kinases (AKs)

Adenylate kinases are ubiquitous enzymes which are found in most cell types and catalyse the equilibrium reaction:



Adenylate kinases have been discussed as potential enzymatic reporters for immunoassay (Gadow *et al.*, 1984; reviewed Kricka 1995). They potentially offer a number of advantages over conventional alkaline phosphatase or horseradish peroxidase conjugates in that they offer a significant amplification of signal via generation of ATP which can

subsequently be coupled with sensitive bioluminescence detection. To date, however, their use has been limited by a failure to generate enzymatically active antibody conjugates. The use of thermostable adenylate kinases was considered as an alternative because enzymes isolated from thermophilic organisms tend to have the same characteristics as their derivative organism and are therefore potentially more robust than their mesophilic counterparts (Vieille & Zeikus, 2001).

Thermophiles are the name for organisms that can survive at temperatures above 45 °C and many have been isolated from extreme environments such as hot springs or volcanoes. Thermophiles are further classified by their optimal temperatures with further categories termed extreme thermophiles or hyperthermophiles with optimum temperatures for growth in excess of 80 °C. Thermophiles including hyperthermophiles are bacterial or archaeal in origin. The archaea are a group of microorganisms which are distinct from bacteria and single celled prokaryotes. It was originally thought that these organisms were relatively rare and found mainly in extreme environments; however they have since been identified in many common habitats. Naturally, enzymes contained within thermophiles have a similar thermal profile in order for the organism to survive. Consequently enzymes isolated from these organisms have found many uses, particularly in the biotechnology industry. For example, thermostable DNA polymerases have been utilised as part of the polymerase chain reaction (PCR) due to a requirement to survive throughout repeated heat steps to >90 °C. The most common DNA polymerase used for this purpose was isolated from a thermostable bacterial species *Thermus aquaticus*, with one from *Pyrococcus furiosus* a recent addition. In addition to extreme heat, some thermophiles and hyperthermophiles require other unusual growth conditions such as the use of sulphur for respiration with the production of sulphuric acid as a by-product. These organisms are therefore adapted to growing in very low pH conditions as well as at high temperatures. Proteins and enzymes isolated from these organisms tend to be robust and can survive extremes of temperature

and pH compared to proteins isolated from mesophilic organisms which have optimal growth conditions at temperatures ranging from 25-40 °C (Brock, 1978). A great number of potential uses of robust enzymes have been identified by researchers and there has been a growing interest in thermophiles. However they tend to have complex growth requirements, particularly due to different requirements for temperature combined with extremes of pH, pressure etc depending on the original source of the microorganism which can make them relatively hard to cultivate within laboratory conditions.

A number of studies have examined the differences between proteins and enzymes isolated from mesophilic and thermophilic organisms in an attempt to identify the differences which confer thermostability. In the case of AKs, one theory is that the enzymes from thermophiles are more rigid than those from mesophiles (Vieille & Zeikus, 2001). However, many enzymes from hyperthermophiles are more active than the equivalent mesophilic enzymes, even at moderate temperatures of ~37 °C (Vieille *et al.*, 2003), conflicting with the theory that the enzymes are more rigid, a characteristic which might be expected to reduce catalytic rate. Differences in amino acid composition have also been suggested (Argos *et al.*, 1979). However despite numerous studies which have taken place particularly recently within this field, no single mechanism has been identified which explains the stability of enzymes isolated from thermophilic and hyperthermophilic organisms, however it is clear that this property has many potential uses especially within biotechnology.

A number of AKs from thermophiles have specifically studied and a recombinant *Bacillus stearothermophilus* AK was produced and compared to *E coli* AK to further elucidate the structural and biochemical differences (Glaser *et al.*, 1992). One of the key findings from this study was that this AK included a tightly bound zinc atom as part of its structure, which was not found in the mesophilic AK and it was hypothesised that complex with zinc

confers a rigidity to the structure of this AK which may be a contributory factor in the thermostability of this protein. Interestingly the *Bacillus stearothermophilus* AK was also shown to be more resistant to trypsin digestion than *E. coli* AK. The role of zinc in the conferring stability of certain AKs has also been studied in the case of a different thermophile, *Thermotoga maritima* with some suggestion that its removal results in loss of thermostability (Vieille *et al.*, 2003). This is interesting, however since not all thermophilic AKs have this structural zinc atom, it is clearly not the only explanation in terms of conferring thermostability.

Adenylate kinase isolated from the hyperthermophile *Sulfolobus acidocaldarius* has also been extensively studied and characterised (Kath, Schmid & Schafer, 1993). AKs from eukaryotes and eubacteria have been shown to have similar sequence and structure (Vonnheim *et al.*, 1998). It was therefore assumed that *Sulfolobus acidocaldarius* AK therefore would also show a high degree of conserved sequences across different species. However, significant differences between the structure of the *Sulfolobus acidocaldarius* AK compared to mesophilic AK and also *Bacillus stearothermophilus* AK, were revealed and it was concluded that this was a novel class of AKs. In addition it was identified that this enzyme is trimeric whereas the majority of other AKs from hyperthermophiles are monomeric (Vonnheim *et al.*, 1998). This trimer structure confers rigidity at the central hydrophobic interface and also features extended β sheet formation at this point, which is specific for *Sulfolobus acidocaldarius* AK. It has been demonstrated that weakening of the interaction of the monomer subunits had a detrimental effect on the thermostability of the enzyme (Backmann *et al.*, 1998; Bönisch *et al.*, 1996).

It has been found that several AKs derived from archaeal origin show similar sequence homology, particularly methanococcal AKs and that these AKs are also trimeric in structure and also contain the extensive β sheet formation at the central interface (Ferber *et*

al., 1997). This has also been identified as the case for *Sulfolobus solfataricus* (Okajima *et al.*, 2002). However a further study (Criswell *et al.*, 2003) reports that the trimeric structure alone is not responsible for thermostability, but that hydrophobic interactions are the basis for thermostability. Studies on chimeric enzymes produced from the genus methanococcus (Criswell *et al.*, 2003) designed with substitutions at the C or N terminal regions, found that it was possible to substantially alter the thermostability of the AKs by amino acid substitution and this may therefore be a useful strategy for the alteration of enzymes to increase thermostability.

A monomeric AK derived from the hyperthermophile *Thermatoga neapolitana* has also been studied (Vieille *et al.*, 2003). This AK has been shown to be stable up to temperatures in the region of 100 °C but has also been shown to be highly active at mesophilic temperatures of ~30 °C. *Thermatoga maritima* has also been shown to be not only active but highly active with the specific activity showing a 10 fold improvement on mesophilic AK such as *E. coli* AK.

There have been studies reviewed in (Vieille & Zeikus, 2001) which have suggested that protein engineering could accomplish an increase in thermostability of enzymes without a reduction in activity. This is based on the results of a number of studies which have shown that thermophilic enzymes tend not to be optimal for activity whereas mesophilic enzymes are less than optimal in terms of stability and it is postulated that this is probably due to lack of evolutionary pressure. This therefore leaves the potential that these enzymes could be further enhanced and optimised for specific uses, by the use of protein engineering.

There is therefore considerable potential for use of AKs from these sources for the purposes of the development of an ultra-sensitive assay format using AK as a reporter enzyme. This exploits the robust qualities of the enzyme to enable conjugation to take

place without loss of enzyme activity and thermostable properties for removal of background signal resulting from AKs present from other sources. In addition, increasing knowledge within this area is opening up possibilities of methods of protein engineering to further increase activity and thermostability thereby substantially increasing the potential of any developed assay (Criswell *et al.*, 2003).

2 Objectives of the Study

2.1 Development of a novel ultra-sensitive immunoassay for the detection of prion protein on surgical instruments

The aim of this study was to develop an ultra-sensitive method for the detection of prion protein adhering to the surface of surgical instruments in order to detect residual contamination of prions *in situ* for the purpose of validation of decontamination methodologies. A novel antibody linked to thermostable AK coupled to ATP bioluminescent detection was therefore proposed to address these requirements (Figure 2-1).

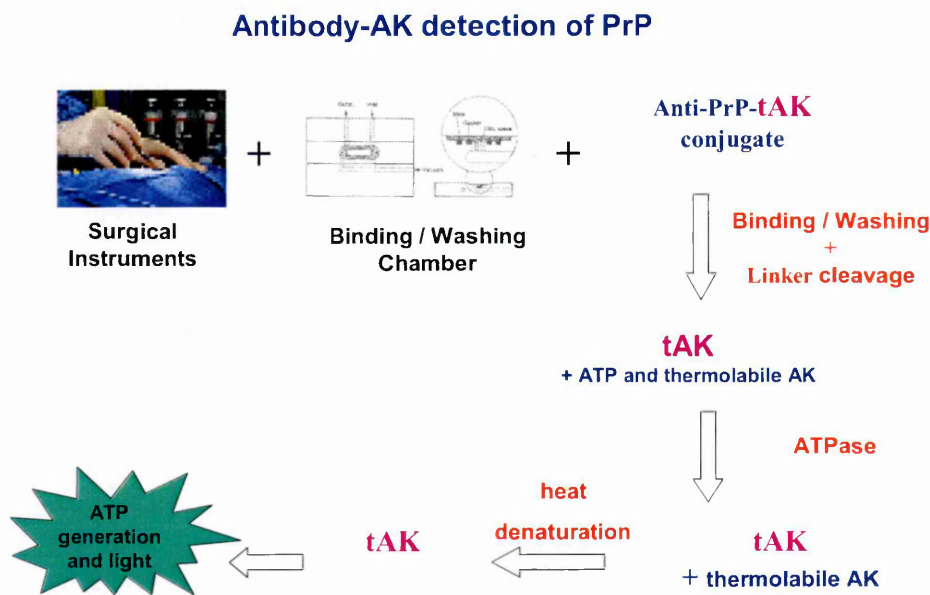


Figure 2-1 Diagram of the proposed ultra-sensitive Prion ELISA.

The assay allows ultra-sensitive detection of material via coupling of ATP production by adenylate kinase to bioluminescence. The assay is designed to allow separation of the antibody from enzyme following binding / washing phases and the inactivation of any contaminating enzyme activity by heat denaturation. The flexible format allows specific issues concerned with detection of prion proteins to be addressed.

2.1.1 Variant Creutzfeldt-Jakob Disease

Creutzfeldt-Jakob Disease (CJD) is a relatively rare form of a human neurodegenerative disorder presenting as either a familial or sporadic disease at a frequency of approximately 1 case per million population. The emergence of a new variant form (vCJD) as a novel form of the disease predominantly in a younger age group, thought to be due to consumption of bovine spongiform encephalopathy (BSE)-infected meat products (Bruce *et al.*, 1997; Collinge *et al.*, 1996), has raised the possibility of a large increase in the numbers of cases. These factors have important public health consequences as there is potential for iatrogenic transmission through routes including surgery, transplants, transfusion and contaminated medical products (Brown 1998; Brown 2001, Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006). Detection of infective material and diagnosis of infected individuals remain major problems in dealing with Transmissible Spongiform Encephalopathy (TSE) diseases both in humans and animals.

To address some of the issues raised by vCJD and in relation to the established routes of iatrogenic spread, a sensitive assay is required for the detection of prion material. The development of the assay aimed to focus on making it applicable to detection of prion material in complex biological materials (e.g. tissue attached to surfaces, blood etc) and its use to validate cleaning and decontamination routines for surgical instruments.

2.1.2 Development of a model assay

In order to develop this assay it was decided that due to the fact that the suggested approach was novel, with no published precedent that development of a model would be advantageous. The aim was to establish the feasibility of the proposed immunoassay and

specific parameters using inexpensive and readily available reagents. It would also avoid the requirement for biological containment at early stages of the assay development and allow optimisation of the assay without the use of infectious material.

2.1.3 Production of anti-PrP polyclonal antisera

It has been identified that prions are poorly immunogenic and there are difficulties associated with development of disease specific antibodies due to the nature of the infectious agent (Prusiner, 1998). Therefore it was important to explore methods for improving the titre and specificity of antibodies produced. The plan was to evaluate the use of a combination of published methods (De Silva, Egodage & Wilson, 1999; Wopfner *et al.*, 1999) to develop a range of polyclonal antisera with different specificities using a range of prion mimetic peptides and a strategy designed to boost the immune response. It was hoped that exploration of a number of different approaches would enable production of stock of anti-PrP antibodies in order to convert the initially developed model assay to an immunoassay specific for detection of prions.

2.1.4 Thermostable adenylate kinases

It was proposed to use an available resource (CEPR Thermophile Culture Collection) as this would enable screening and selection of thermostable adenylate kinases for further characterisation and determination of the presence of suitable qualities for use in the developed immunoassay. It was anticipated that once potentially suitable AKs were identified that work would focus on development of a method of producing a stock and therefore the use of recombinant DNA technology would be explored. Once a recombinant

version of the selected AK was produced, the next phase was for methods of purification of AKs to be established based on the results of the characterisation studies.

2.1.5 Assessment of detection limits

The aim was that the assay would be fully optimised prior to work with prion infective material. Therefore detection limits for rec PrP would initially be assessed alone and followed by specific detection within various biologically relevant substrates such as neuronal tissue, blood and sera. This would be followed by assessment for potential detection of rec PrP on the surface of surgical instruments and the possibility of designing a format suitable for validation of decontamination processes within sterile service departments (SSDs). The aim was to utilise the results obtained in all the initial experiments to transfer to working with infectious prion material within suitable containment facilities and thereafter repeat the evaluation of the AK-ELISA for sensitive detection of prion infective material.

It is clear from the published literature that there still remain considerable gaps in the current scientific knowledge on the nature of the prion infectious agent. It was therefore hoped that an increased understanding would be gained during the course of the study on some of the scientific issues specific to this very unusual infectious agent.

3 Development of a model thermostable AK immunoassay

3.1 Introduction

The aim was to demonstrate the feasibility of the proposed ultra-sensitive detection method for prion protein by the establishment of a model system. The assay required design of a number of unusual features to make it applicable to the specific problems associated with detection of CJD on the surface of surgical instruments. The developed assay therefore required:

- To be ultra-sensitive to address the sensitivity problems associated with detection of prion infective material.
- To be able to detect prions within complex mixtures of biological material such as neuronal tissue or blood without compromise of the assay sensitivity.
- The flexibility to allow the potential separation of the antibody binding / washing and assay phases to enable specific detection of prion contamination on the surface of surgical instruments.

Additionally, in order for the assay to be routinely adopted within a clinical setting, the developed method had to be practical, relatively cost effective and applicable to standard hospital laboratories. There were a number of parameters proposed for the assay design, which had no precedent in published literature and therefore development of a model assay version allowed the various stages of the assay to be trialled using relatively inexpensive and readily available reagents. It also avoided the requirement for biological containment at early stages of the assay development by allowing establishment of the assay format without the use of infectious material.

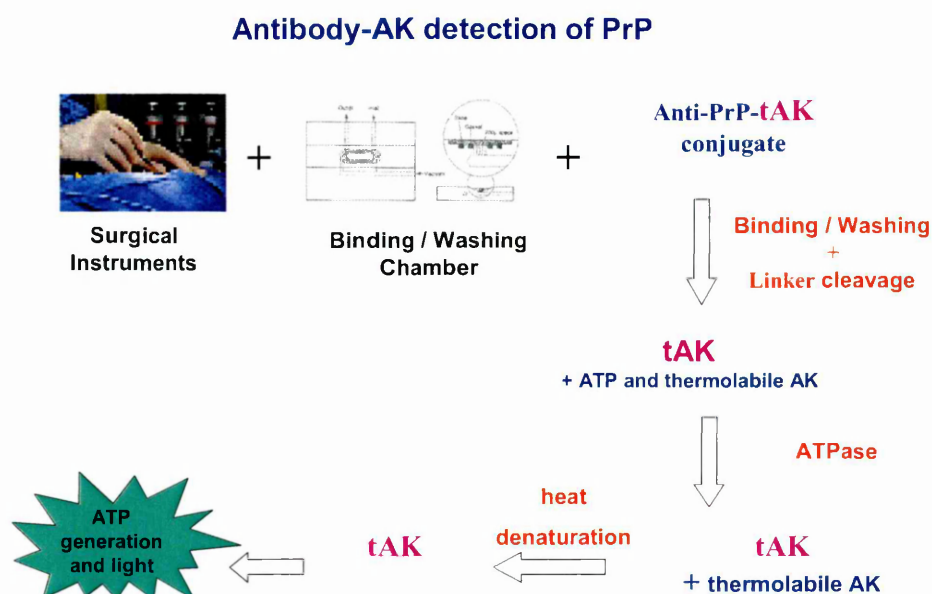
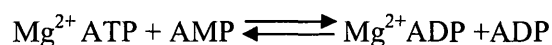


Figure 3-1 Diagram of the proposed ultra-sensitive Prion ELISA.

The assay aims to provide ultra-sensitive detection of prion infective material on surface of surgical instruments by coupling of ATP production by a thermostable adenylate kinase to bioluminescence. The assay is designed to allow separation of the antibody from enzyme following binding / washing phases and the inactivation of any contaminating enzyme activity by heat denaturation. The flexible format aims to address specific issues concerned with detection of prion proteins.

Figure 3-1 shows a schematic of the proposed assay format for detection of prion proteins on the surface of surgical instruments. The diagram demonstrates the use of thermostable adenylate kinase (AK) conjugated to an anti-PrP antibody via a cleavable linker which would enable separation of the initial antibody binding step and allow the remaining steps of the assay to be carried out using standard laboratory equipment. The inclusion of heat steps and incubation steps with ATPase are designed to remove all potentially contaminating AK and ATP prior to generation of ATP by the thermostable AK and bioluminescent detection. For the purpose of development of a model system the main focus was to firstly establish whether conjugation of the thermostable AK to an antibody

was possible without loss of activity of the enzyme. Secondly was that the AK could be subsequently cleaved and used to produce ATP which could be coupled to bioluminescent detection. AKs are ubiquitous enzymes which are found in most cell types and catalyse the equilibrium reaction:



The normal direction of the reaction for the purposes of cellular metabolism is in the direction of formation of ADP and AMP, however in the presence of an excess of ADP supplied as a substrate, the reaction can be driven in the opposite direction resulting in production of ATP which can be detected using bioluminescence (Blasco *et al.*, 1998).

The development of this model assay therefore aimed to establish 'proof of principle' of the AK assay and specifically of;

- Use of ADP as substrate for the production of ATP from bound thermostable adenylate kinase (AK) and subsequent bioluminescence detection.
- Successful conjugation of thermostable AK to an antibody molecule without loss of biological activity.
- Cleavage of the AK moiety without loss of biological activity to allow separation of antibody binding and detection if required and demonstration that detection of antigen using this methodology was possible.

3.2 Materials and Methods

3.2.1 Comparison of different sources of ADP

A 1:10,000 dilution of *Bacillus stearothermophilus* AK (myokinase) (Sigma) was obtained and prepared as a stock solution in phosphate buffered saline (PBS) and 100 µl added to microtitre plate wells (Nunc-Immuno™ MaxiSorp™) with the exception of the controls. Two commercial samples of adenosine 5'-diphosphate (ADP) were obtained as follows: from a bacterial source (Roche) and equine muscle (Sigma) and prepared as 0.3 mM solutions in magnesium acetate buffer (15 mM magnesium acetate buffer + 1 mM ethylenediaminetetraacetic (EDTA), pH 6.7). Titrations of ADP were prepared by doubling dilutions in magnesium acetate buffer and 100 µl of each dilution was added to appropriate wells and incubated at room temperature for 30 minutes. 30 µl of Luciferin-luciferase (L/L) reagent (ATP Kit SL) (Biothema) was added to each well and read immediately in a plate luminometer (Berthold Orion) measuring relative light units (RLUs).

3.2.2 Comparison of the inherent ATP contamination

A further commercial source of ADP was obtained which was AK & Adenosine 5'-triphosphate (ATP)-free ADP (Celsis). 0.3 mM dilutions of all three ADP (Roche, Sigma, Celsis) were prepared in magnesium acetate buffer and 100 µl of each dilution was added to appropriate wells. 30 µl of Luciferin-luciferase reagent (ATP Kit SL, Biothema) was added to each well and read in a plate luminometer immediately.

3.2.3 Purification of IgG

50 mL of polyclonal rabbit serum produced to Keyhole Limpet Haemocyanin (KLH) was obtained from the Biological Investigations Group (BIG), CAMR (no information was available on the method of production). 5 mL of sera was diluted to 20 mL with 20 mM potassium phosphate buffer, pH 7.0 and small particles removed by filtration using a Millipore filter (0.4 μ). A Protein G Sepharose Fast Flow column (Pierce) was pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.0 using Fast Protein Liquid Chromatography (FPLC) (Pharmacia) and sample applied. The column was washed with the above buffer until the absorbance (280 nm) had returned to baseline. 2.5 mL fractions of purified IgG was eluted by addition of 100 mM Glycine, pH 2.5 and neutralised immediately in phosphate buffered saline (PBS). Peak fractions were identified using the chart recorder trace, collected, pooled and subsequently dialysed against phosphate buffered saline (PBS) (20 mM Phosphate buffer, 150 mM sodium chloride (NaCl), pH 7.6).

3.2.4 Calculation of titre of purified IgG

The wells of a 96 well Microtitre plate (Nunc-Immuno™ MaxiSorp™) were coated by addition of 100 μ L per well of KLH (Sigma) diluted to 1 μ g.mL⁻¹ in coating buffer (0.05 M sodium carbonate buffer, pH 9.6) and incubated overnight at room temperature. Plates were washed three times in PBS containing 0.05 % Tween 20 (PBS-T) using a Multiskan AC Plate Washer and non-specific binding blocked by the addition of 300 μ L per well of a solution of 3 % dried milk (Marvel) diluted in PBS and incubated for 1 hour at 37 °C. The solution was removed by washing three times in PBS-T. The post purification antiserum was diluted from 1:250 by doubling dilutions in PBS and incubated for 1 hour at 37 °C.

The solution was removed by washing wells with PBS-T as previously described, followed by addition of 100 μ l per well of a 1:20,000 dilution of anti-rabbit IgG peroxidase conjugate (Sigma) and incubated for 1 hour at 37 °C. Following incubation the wells were washed as previously described and then 100 μ L per well of tetramethlybenzidine (TMB) liquid substrate (Sigma) was added and incubated for 15 minutes at room temperature. Colour development was stopped by the addition of 100 μ L per well of 1M sulphuric acid. absorbance (450 nm) read on a Multiskan plate reader (Labsystems).

3.2.5 Estimation of protein concentration

Coomassie Plus Protein Assay reagent (Pierce) was used by adding 50 μ l of each sample / standard to a tube and adding 1.5 mL of Coomassie Plus Reagent, mixing and incubating at 37°C for 30 minutes before reading the absorbance (595nm) in a spectrophotometer (Labsystems). A standard curve was created by using a range of bovine serum albumin (BSA) standards supplied with the kit to calculate known values at 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 mg.mL⁻¹. The protein concentration of the sample was calculated with reference to the standard curve.

3.2.6 Concentration of the IgG sample

Concentration of the purified IgG samples was achieved by ultra-filtration using Ultrafree-15 (Millipore) 10 kDa filter. Each filter was loaded with 10 mL of the protein solution and samples spun at 3000 rpm in a microcentrifuge for 20 minutes and the process repeated twice (3 x 20minutes). When concentration to approximately 1.5 mL was achieved a further 5 mL of sample was added to each filter and the procedure repeated until required.

3.2.7 Comparison of thermal stability of AKs

Two samples of AK (myokinase) were obtained, one isolated from a thermophilic microorganism *Bacillus stearothermophilus* (Sigma) and the second isolated from rabbit muscle (Sigma) and diluted to an initial starting concentration of enzyme activity of 1 unit.mL⁻¹ (one unit of specific activity is defined as that producing 1 μ mol of ATP.min⁻¹ at 70 °C) and then diluted. 100 μ l of each preparation was added to wells in a microtitre plate. The plate was heated to 40, 50, 60 or 70 °C for 10 minutes followed by readjustment to 37 °C in a thermocycler (Techne). 100 μ l of 0.13 mM of ADP (Celsis) in magnesium acetate buffer was added diluted as before and incubated for 30 minutes at 37 °C. Production of ATP was measured as RLU in the plate luminometer by the addition of 30 μ L of luciferin-luciferase reagent (Biothema).

3.2.8 Conjugation of AK to purified IgG

Adenylate kinase (Myokinase) isolated from the thermophilic bacteria *Bacillus stearothermophilus* (Sigma) was diluted to a concentration of 10 mg.mL⁻¹ in PBS (20 mM phosphate buffer, 150 mM sodium chloride (NaCl), 1 mM EDTA pH 7.5). The heterobifunctional reagent, N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pierce) was used for the conjugation reaction at 10-fold molar concentration of SPDP to protein. A 4.1 mM concentration of SPDP was required to derivatise AK prior to conjugation to IgG. This was achieved by dissolving 2.6 mg of SPDP in 20 μ l of anhydrous dimethylsulphoxide (DMSO) followed by the addition of 10 μ l of this solution drop wise to the dissolved AK and mixed for 1 hour at room temperature using a magnetic stirrer.

The derivatised solution was transferred to pre-prepared dialysis tubing (VWR) and dialysed extensively against PBS overnight at 4 °C to remove unreacted SPDP. 12 mg of dithiothreitol (DTT) was dissolved in 500 µl of PBS and 250 µL added to the SPDP derivatised AK and incubated at room temperature for 30 minutes while mixing continuously using a magnetic stirrer. The resultant solution was dialysed against PBS to remove DTT overnight at 4 °C. 500 µL of the stock solution of purified anti-KLH IgG (20 mg.mL⁻¹) was diluted with 500 µL of PBS to a final concentration of 10 mg.mL⁻¹ and derivatised with SPDP as described above. Unreacted SPDP was removed by passing the solution through a PD10 column (Pierce) pre-equilibrated with PBS. 1 mL fractions were collected and the presence of IgG determined by measurement of the absorbance (280 nm) using a UV spectrophotometer and quartz cuvette. The fractions containing the protein were pooled and concentrated by ultra-filtration as described in section 3.2.6. Two ratios of IgG to AK which were 2:1 and 1:2 respectively were calculated and conjugated by addition of the required amount of modified IgG to derivatised and reduced AK followed by incubation overnight while mixing at room temperature. The conjugation reaction was stopped by the addition of 10 µL of a 5 mg.mL⁻¹ solution of N-ethyl maleimide (Sigma) that was freshly prepared in anhydrous DMSO followed by incubation for 30 minutes at room temperature. The unreacted N-ethyl maleimide removed by dialysis against PBS.

3.2.9 Assessment of conjugation by HPLC gel filtration

A TSK2000 HPLC gel filtration column was pre-equilibrated with PBS and calibrated by the addition of the following standard preparations; cytochrome C (bovine heart), bovine serum albumin (BSA) and alcohol dehydrogenase (ADH) obtained from Sigma UK. A standard curve was prepared based on the retention time in minutes of the known molecular weight (kDa) and this was used to determine the molecular weights of unknown

samples. After the last stage of the conjugation procedure (modified AK and IgG were combined as described in section 3.2.8), the reaction was followed by loading 20 μ L samples of the conjugation preparation onto the pre-equilibrated and calibrated TSK 2000 column at different time intervals throughout the conjugation process to monitor the reaction and a trace obtained.

3.2.10 DTT cleavage of the conjugate preparation

0.5 mL of the prepared conjugate solution was loaded onto the TSK2000 HPLC gel filtration column and peak fractions were collected manually as described in 3.2.9. 20 μ L of the conjugate was re-loaded onto the column to check the purity of the conjugate fraction prior to DTT (Sigma) cleavage. DTT cleavage was achieved by incubating the conjugate preparation with 25 mM DTT for 30 minutes at room temperature. Cleavage of the conjugate preparation to derivative fractions of IgG and AK was monitored by loading 20 μ L samples onto the column and peak fractions collected manually as before and a trace of the reaction obtained.

3.2.11 Detection of KLH by anti-KLH-AK conjugate

A microtitre plate was coated with KLH and blocked as described in section 3.2.4. 100 μ L per well of anti-KLH-AK conjugate was added from a starting dilution of 1:250 in PBS and diluted using doubling dilutions. Following incubation for 1 hour at 37 °C and then wells washed as previously described. 100 μ L of 0.3 mM ADP was prepared in magnesium acetate buffer and added to the appropriate wells and incubated for 30 minutes at 37 °C. 30 μ L of luciferin-luciferase reagent (Biothema) was added and the results read immediately

by plate luminometer (Orion Berthold). Controls wells which omitted antigen; antibody-AK conjugate and ADP respectively were included within the assay.

3.3 Results

3.3.1 *Bioluminescent Detection*

In order to produce a model bioluminescent assay, it was crucial that a method of generating ATP from the bound marker enzyme, thermostable adenylate kinase, was developed. ADP was purchased from a number of commercial sources for assessment and comparison. However initial results using ADP generated ATP, followed by bioluminescent detection routinely showed very high background results, which made the assay unreadable (results not shown). Therefore it looked likely that the non-specific light emission could be due to reagent contamination, for example, contaminating AK present in the luciferin-luciferase reagent and / or ATP contamination of the purchased ADP substrate.

The two initially purchased samples of ADP (Roche and Sigma) were tested directly by ATP bioluminescent assay and the results showed that there was an appreciable quantity of contaminating ATP. Contact was made with a small research company who were developing ATP bioluminescent detection as a direct measure of bacterial contamination on environmental surfaces and who had developed a set of ATP and AK free reagents which they were keen to have assessed for a different use (Celsis, Cambridge, UK) This low-ATP ADP has a contamination level of ATP at 0.1 ppm (compared to ~ 0.2% quoted in the other reagents) which, made it ideal for this purpose and also had been further purified to remove any contaminating adenylate kinase.

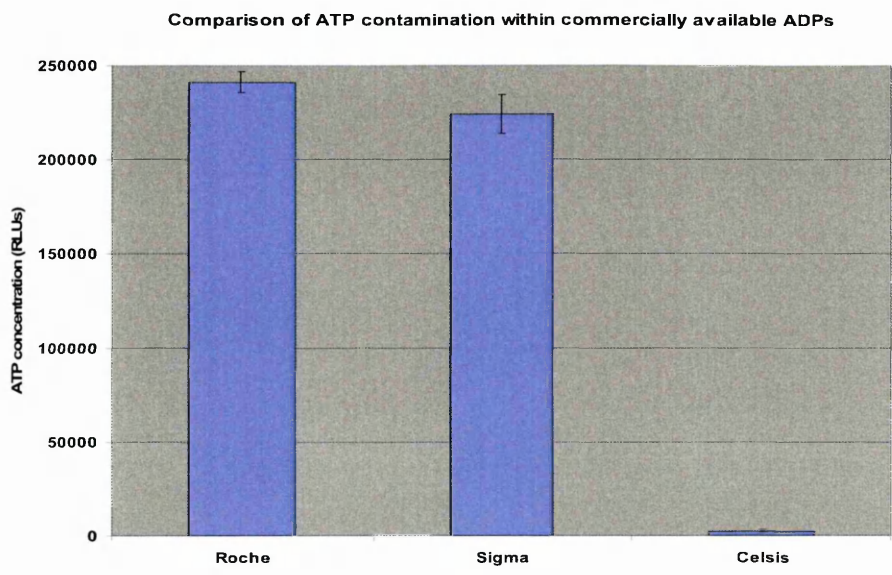


Figure 3-2 Comparison of three different samples of ADP

0.3 mM dilutions of ADP (Roche, Sigma, Celsis) were prepared in magnesium acetate buffer and 100 µl of each dilution added to appropriate wells. 30 µL of Luciferin-luciferase reagent (ATP Kit SL) (Biothema) was added directly to each well and the results read in a plate luminometer (Berthold Orion) immediately (RLUs).

Figure 3-2 shows the results of the ATP bioluminescent assay which was carried out directly on the three samples of ADP reagent to determine the actual degree of ATP contamination present within the reagents (3.2.2). The results demonstrated that two of the purchased samples of ADP substrate (Roche and Sigma) both contained significant levels of ATP contamination, whereas the ADP substrate from Celsis was of sufficient purity to solve the problem of high background signal generated from presence of inherent ATP. It was clear therefore that a sample of ATP-free ADP was critical for the development of a model assay using thermostable AK as a marker enzyme.

3.3.2 Calculation of titre of purified anti-KLH IgG

IgG was purified using the methodology described in Section 3.2.3 and the titre of the anti-KLH assessed by ELISA to determine if the antibody had sufficiently high titre to be useful for conjugation to thermostable AK for use in the model assay (3.2.4).

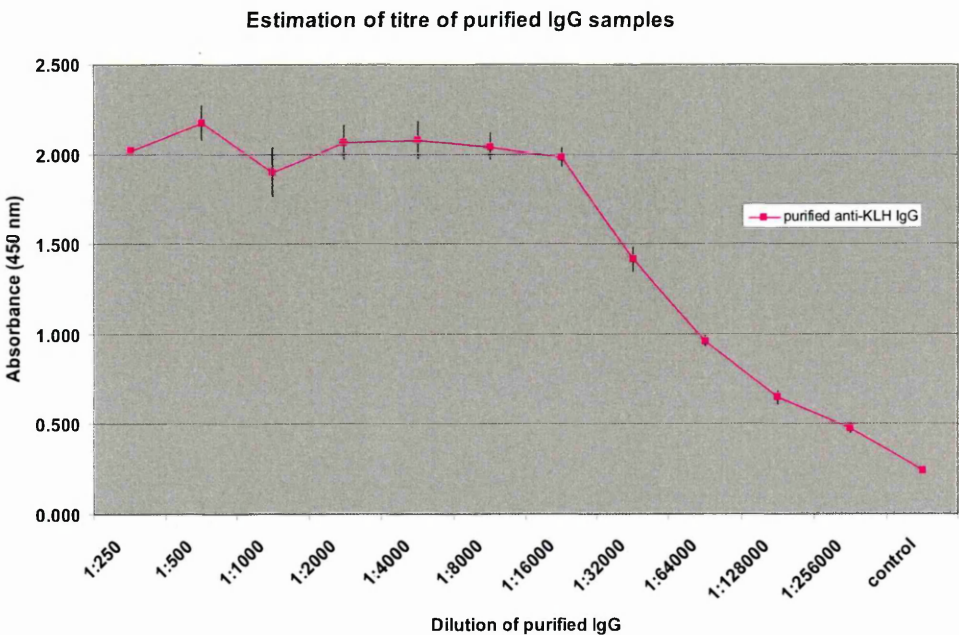


Figure 3-3 Calculation of the titre of anti-KLH IgG purified from polyclonal antisera.

A microtitre plate was coated, blocked and samples added as described in section 3.2.4. Post purification antisera were diluted from 1:250 dilution and incubated for 1 hour at 37 °C. The presence of bound antibody was detected using a secondary peroxidase labelled antibody followed by colourimetric detection using tetramethylbenzidine (TMB) substrate and measurement of absorbance (450 nm).

The results demonstrated that the purified fraction of anti-KLH IgG had a titre of approximately 1:16,000 - 1:32,000, and therefore was suitable for use in the model assay and conjugation to thermostable AK.

3.3.3 Estimation of protein concentration of purified anti-KLH IgG

The protein concentration was estimated to be in excess of 1 mg.mL^{-1} as described in section 3.2.5. However, in order to improve the success of the conjugation chemistry, it was desirable that the protein concentration should be in the region of 10 mg.mL^{-1} and therefore the purified IgG was further concentrated by ultrafiltration (3.2.6). Two samples of concentrated purified IgG were obtained which contained approximately 20 mg.mL^{-1} and stored frozen at -20°C until used.

AK (myokinase) isolated from the thermophile *Bacillus stearothermophilus* (Sigma) was purchased for assessment for suitability as the thermostable marker enzyme for conjugation to antibody to establish 'proof of principle' for the methodology. In order to determine if this purchased stock of adenylate kinase had the correct properties for use in the model assay, a comparison of the thermostability of this enzyme was carried out with AK isolated from a different source of AK relevant to potentially contaminating tissue and therefore rabbit muscle AK was selected for this purpose (Sigma). The two sources of AK were compared for thermostability (3.2.7) by heat treating at temperatures ranging from $37\text{-}70^\circ\text{C}$ for 10 minutes before incubating with ADP substrate followed by a standard bioluminescent assay and the results are shown in Figure 3-4.

3.3.4 Comparison of thermostability of AKs

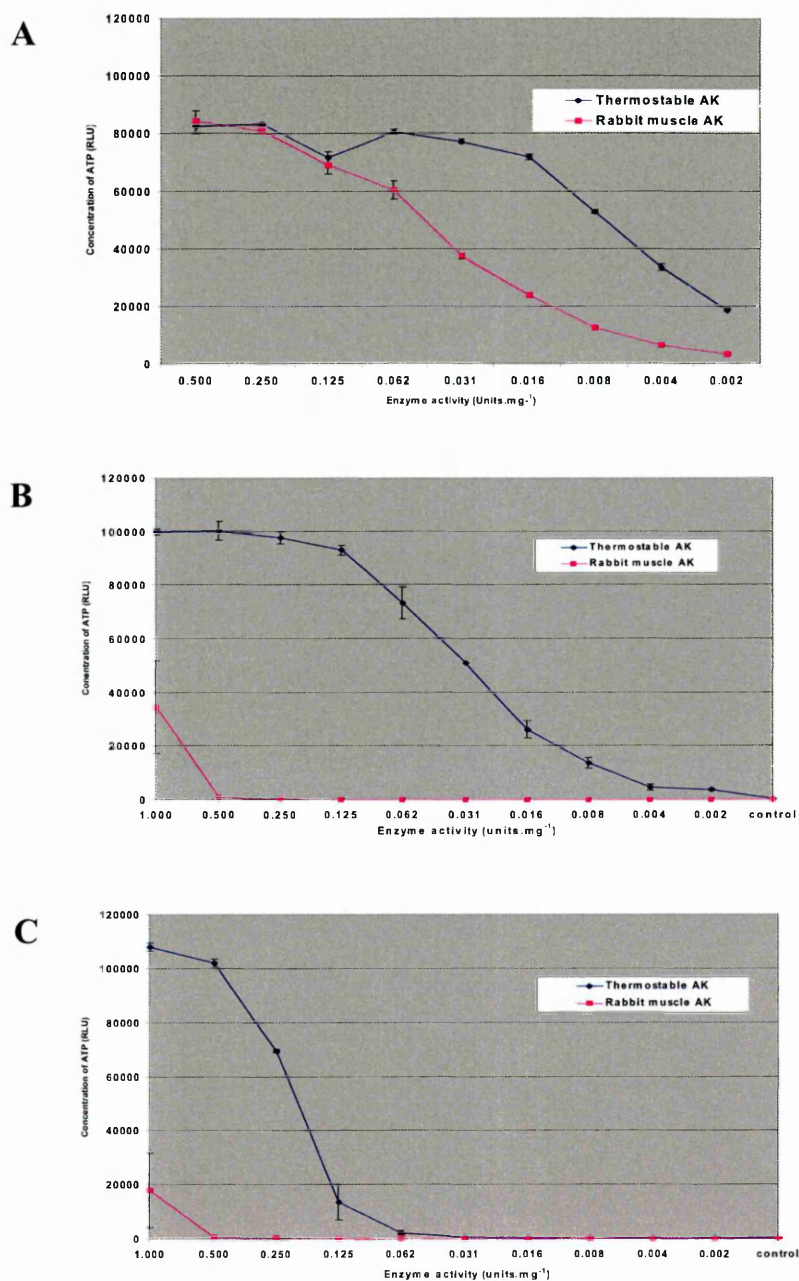


Figure 3-4 Comparison of thermostability of two samples of AKs

Two sources of AK (*Bacillus stearothermophilus* and rabbit muscle) were diluted from same starting concentration. In panels B and C the same dilutions of enzyme were used but were incubated for 10 minutes in the microtitre plate at 60 °C (panel B) and 70 °C (panel C) while panel A had no heat treatment, prior to incubation with ADP and detection of ATP by bioluminescence assay.

The results showed a significant difference in thermostability of the two sources of AK. *B. stearothersophilus* adenylate kinase was shown to be stable and active at temperatures significantly higher than required to inactivate conventional adenylate kinases (e.g. rabbit muscle adenylate kinase). Figure 3-4 shows comparison of the activities of AKs isolated from rabbit muscle AK and *B. stearothersophilus* with or without incubation at 60 or 70 °C. The results of this assay demonstrated that *B. stearothersophilus* AK was thermostable and therefore suitable for use in the model assay. However it was noted that at 70 °C, there was considerable reduction (~10-fold) in the activity of *B. stearothersophilus* AK and therefore a more thermostable AK would be essential for the final assay format as it was anticipated that heat steps of at least 70 °C would be required, especially if detection of prion infectivity was to be achieved within a biological material such as neuronal tissue, blood or sera where a significant contamination of thermolabile AK would be expected.

3.3.5 HPLC analysis of conjugation reaction

A number of antibody-enzyme conjugates were prepared using the heterobifunctional reagent SPDP (3.2.8). The method was evaluated and optimised to a ratio of antibody to enzyme of 1:3 respectively. The conjugates showed a peak at approximately 200 kDa by fractionation by HPLC gel filtration which was consistent with the expected molecular mass of the antibody-AK conjugate (Figure 3-5).

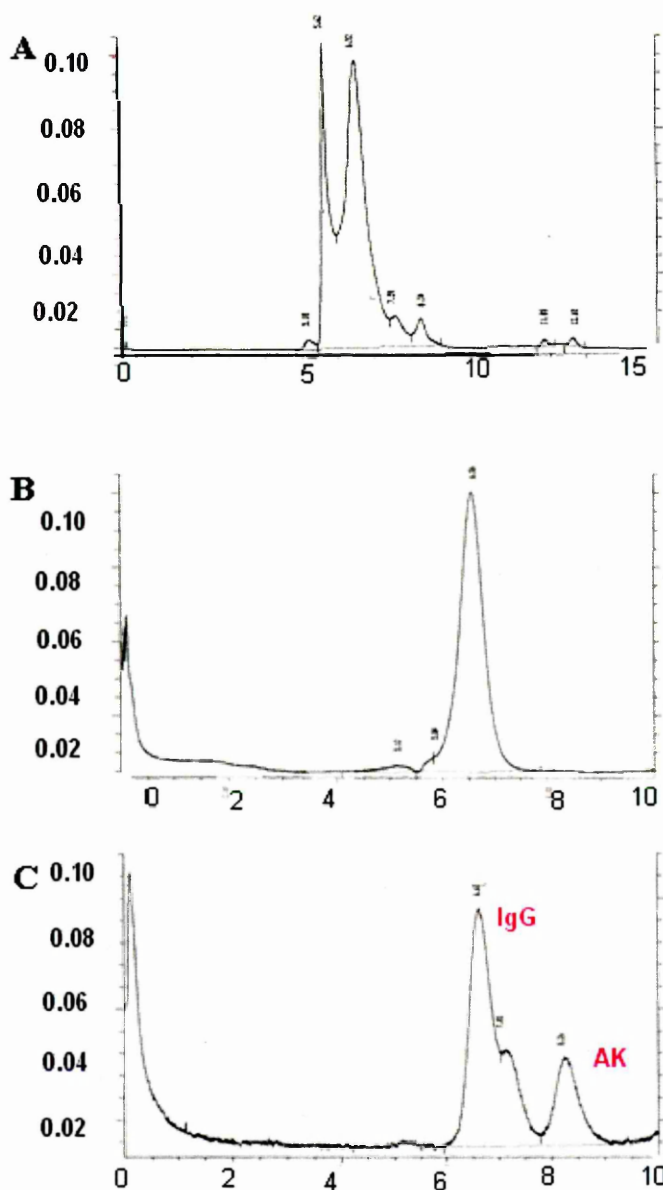


Figure 3-5 Characterisation of anti-KLH AK conjugates by HPLC.

Purified anti-KLH IgG was conjugated to *B. stearothermophilus* AK using SPDP. The process resulted in the formation of stable IgG-AK conjugates as demonstrated by size fractionation on an HPLC column (panel A). Conjugates were purified (panel B). Panel C shows the cleavage of the conjugates into constituent IgG and AK fractions as labelled, by reduction with DTT demonstrating that the molecules can be cleaved to allow the release of the enzymatic activity. X-axis shows retention time (minutes) and Y axis shows absorbance 280nm. Note the retention times for the HPLC column differ between panel A and panels B and C.

The purified conjugate was demonstrated to be cleavable by incubation with 25 mM dithiothreitol (DTT) for 30 minutes to yield active enzyme and the subsequent bioluminescence reaction to be unaffected by this concentration of DTT (3.2.10). These results demonstrated that the *B. stearrowthermophilus* AK could be used to generate antibody enzyme conjugates without loss of biological activity unlike previously reported results with mesophilic AK, (Gadow *et al.*, 1984).

3.3.6 Detection of KLH with anti-KLH IgG-AK conjugate

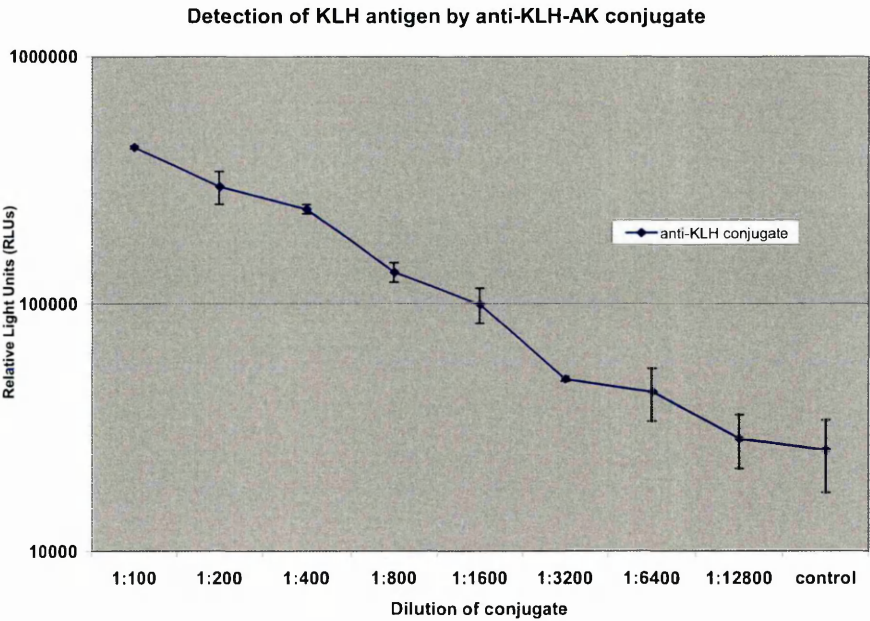


Figure 3-6 Model AK ELISA -detection of KLH antigen by anti-KLH-AK conjugate.

Microtitre plate wells were coated with KLH at a concentration of $1 \mu\text{g.mL}^{-1}$. The KLH antigen was detected by the addition of serially diluted anti-KLH-AK conjugate which was followed by incubation with ADP after washing of the wells with PBS-T. The ATP produced was measured as RLU in a plate luminometer by the addition of 30 μL of L/L reagent.

The anti-KLH IgG-AK conjugate preparation was assessed by AK-ELISA to determine if successful detection of the KLH antigen by the use of the model assay format could be achieved (3.2.11). Several parameters of the assay were optimised prior to this assay and included increasing the length and temperature of the incubation with ADP and this resulted in a positive result. This was demonstrated by a titration curve in response to dilution of the antibody-enzyme conjugate thereby demonstrating detection of KLH at a coating concentration of $1 \mu\text{g.mL}^{-1}$ (Figure 3-6).

3.4 Discussion

The aim was to develop a model to demonstrate 'proof of principle' of the proposed assay in order to demonstrate that various parameters of the assay were feasible such as successful conjugation of the antibody to AK without the loss of the biological activity of the enzyme and to prove that AK could be coupled with bioluminescent detection technology.

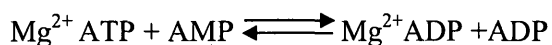
This was particularly important as the ultimate aim of the assay was detection of BSE 301V infectious mouse brain, or CJD which would necessarily involve biological containment. Hence the assay could be trialled and optimised without the requirement for containment at the early stages of development.

ATP bioluminescence has been shown to offer a sensitive detection methodology and has been extensively used for bacterial detection within hygiene monitoring (reviewed Kricka, 1995). The potential use of AK labelled antibody coupled with bioluminescent detection by generation of ATP has been recognised previously, however conjugation of a mesophilic AK to an antibody had resulted in the loss of biological activity of the enzyme

(Gadow *et al.*, 1984). The plan was to use an AK isolated from a thermophilic microorganism as they tend to be more robust with inherent thermostable properties due to the growth conditions of the originating microorganism (Vieille & Zeikus, 2001). It was therefore hoped that the use of a thermostable AK for this purpose would avoid problems associated with loss of enzyme activity with the additional advantage that the heat stability properties could be utilised within the final assay format and enable inclusion of heat steps to effectively remove signal resulting from contaminating AKs. This was important as it was proposed that the assay would enable sensitive detection of prions within biological materials such as tissue or blood. It was therefore critical to establish that conjugation of a thermostable AK to an antibody was possible using a potentially more robust enzyme isolated from a thermophilic microorganism, without a resulting loss of enzyme activity or thermostable properties.

It was ultimately hoped that the final assay format would enable detection of prion contamination on the surface of surgical instruments and therefore the assay had to be flexible to allow the potential separation of the antibody binding / washing and assay phases and allow the remaining steps of the assay to be carried out using standard laboratory equipment. Therefore once it was established that conjugation of the antibody to AK could be successfully achieved it then remained to show that cleavage of the AK moiety, without loss of activity, was possible using readily available reagents.

AKs catalyse the equilibrium reaction:



As discussed previously, the normal direction of the reaction for the purposes of cellular metabolism is in the direction of formation of ADP and AMP, however in the presence of an excess of ADP supplied as a substrate, the reaction can be driven in the opposite direction resulting in production of ATP which can be detected using bioluminescence (Blasco *et al.*, 1998).

Researchers have previously described this methodology for the purpose of specific detection of bacteria by lysis using bacterial phages to release intracellular AK followed by incubation with ADP and bioluminescent detection of ATP generated (Blasco *et al.*, 1998; Squirrell, Price & Murphy, 2002). The use of an excess of ADP substrate was therefore chosen as a method of producing ATP from the bound AK and which would hopefully result in amplification of the signal with benefits in sensitivity of detection. The other important assay parameters proposed in the original plan was the combination treatment with ATPase or apyrase followed by inclusion of heat steps to inactivate any contaminating ATP or thermolabile AK present before incubation with ADP. The inclusion of this combination of steps would ensure that the entire bioluminescent signal was a result of generation of ATP by the thermostable AK antibody label and not due to the presence of contaminants, from e.g. biological tissue present. The use of apyrase as a method of reducing contaminating ATP present in biological samples when detecting bacteria for the purpose of hygiene monitoring has been described previously (Sakakibara *et al.*, 1997).

Evaluation of initially sourced commercial ADP showed that there was a high degree of ATP contamination present which rendered them unfit for this purpose as it caused an extremely high background signal which effectively obliterated any results obtained utilising this method. It was obvious then that this was a critical step and good quality reagents were necessary to ensure that no AK or ATP was introduced at this stage, which would be after the heat inactivation and apyrase steps in the proposed model assay. This

was particularly important as one of the requirements of the assay would be a capability of detection of very small amounts of ATP generated from the AK antibody level in order that the assay could be truly ultra-sensitive. It was therefore established as crucial that reagents were free of contamination from either ATP or AK.

Contact was made with a small research company who were developing ATP bioluminescent detection as a direct measure of bacterial contamination on environmental surfaces and who had developed a set of ATP and AK free reagents which, they were keen to have assessed for a different use (Celsis, Cambridge, UK) (Squirrell, Price & Murphy, 2002). The Celsis ADP stated that the contamination level of ATP was 0.1 ppm which made it potentially ideal. A quick comparison of inherent ATP contamination of the three sources of ADP by direct ATP bioluminescence immediately indicated that the use of this ADP reagent would effectively remove the high background signal which was obscuring the results when using all the other commercially available ADP. This therefore enabled the AK ELISA to be further developed and optimised.

In order for the proposed assay methodology to be successful, the properties of thermostability of the AK label would be required to be utilised in order for suitable heat steps to be included in the assay to remove any AK from alternative sources such as contaminating biological tissue. This was particularly important due to the ubiquitous nature of AKs, which are present in most cell types and which therefore could result in high non-specific background generation of ATP. Therefore it vital that the AK used was sufficiently thermostable to withstand significantly higher temperatures than a normal source of AK. *B. stearothermophilus* AK has been previously described as thermostable (Glaser *et al.*, 1992) and the results of this study (3.3.4) demonstrated stability and activity at temperatures of 60 and 70 °C which was significantly higher than temperatures capable of inactivating conventional adenylate kinases (e.g. rabbit muscle adenylate kinase). This

showed that this enzyme was suitable for use to establish the feasibility of proposed assay however it was apparent that although the *B. stearothermophilus* AK enzyme showed reasonable heat stability at 60 °C there was a considerable loss of activity, approximately 10-fold, apparent at higher temperatures such as 70 °C. For development of the final assay therefore, it was identified as desirable that a more thermostable AK should be sourced, to enable inclusion of heat inactivation steps of temperatures of 70 °C and above, in order to effectively remove AK present in potentially substantial amounts of contaminating tissue on the surface of surgical instruments.

The use of a standard antigen-antibody combination was selected for development of the initial model with keyhole limpet haemocyanin (KLH) chosen as antigen. This enabled extensive evaluation of the conjugation methods using relatively inexpensive and readily available reagents. KLH is commonly used as a carrier protein for haptens for the production of antisera, therefore sera produced to alternative haptens using this carrier protein will have high immunoreactivity for KLH. This combined with the readily commercial availability of purified KLH at relatively low prices meant that this was a practical combination for use for this purpose (Harlow & Lane, 1988). The conjugation method was optimised and the reaction followed using HPLC gel filtration and the results demonstrated successful conjugation and the purified conjugate fraction found to have active AK by subsequent bioluminescent detection. These results demonstrated that the *B. stearothermophilus* AK unlike mesophilic AKs, could be used to generate conjugates, which retain biological activity and therefore suitable for this purpose.

Another key stage of development of the assay was to demonstrate that it was possible to cleave the AK moiety from the bound antibody without loss of the biological activity of the enzyme. This is potentially a critical step for applications of the assay where the binding and washing phases need to be carried out *in situ*, e.g. for the validation of surgical

instrument decontamination, to enable completion of the assay using standard laboratory equipment. The conjugate was demonstrated to be cleavable by incubation with dithiothreitol (DTT) to yield active enzyme and the subsequent bioluminescence reaction to be unaffected by the concentration of DTT (25 mM) required to cause linker cleavage. This thereby indicated that it would be possible to include this step in the final assay format without detriment to detection of antigen.

It therefore remained to demonstrate use of the model assay for detection of the antigen selected for the purpose using the thermostable AK immunoassay. A number of assay conditions were further optimised prior to this, including length and temperature of incubation steps and concentration of ADP substrate. The results of the optimised AK-ELISA demonstrated a titration curve in response to dilution of the antibody-enzyme conjugate demonstrating successful detection of KLH at a coating concentration of $1 \mu\text{g.mL}^{-1}$ using the novel methodology and thereby establishing “proof of principle”.

4 Selection of improved thermostable adenylate kinases

4.1 Introduction

A thermostable adenylate kinase isolated from *Bacillus stearothermophilus* was used within development of the model assay to demonstrate the feasibility of the proposed methodology. It was shown that this AK still retained activity at temperatures of up to 70 °C; however it was noticeable that there was substantial loss of activity (~10-fold) at this temperature, which would make its use unsuitable for the final assay format. The ultimate aim of the methodology being designed was to enable sensitive detection of prions within an excess of biological material such as blood and neurological tissue. This would therefore, necessitate inclusion of sufficient heat steps to reduce background signal from contaminating AKs contained endogenously within tissue. This combined with economic reasons due to the commercial cost of the *Bacillus stearothermophilus* AK, made it necessary to an alternative AK with superior thermostable properties which would be able to be produced in-house in sufficient quantities to allow development of the assay.

Enzymes isolated from thermophiles tend to be more robust than their mesophilic counterparts (Vieille & Zeikus, 2001). Furthermore, thermophiles are classified by their optimal temperatures with an additional category termed extreme thermophiles or hyperthermophiles which have optimum temperatures for growth in excess of 80 °C. Proteins and enzymes isolated from these organisms can survive extremes of temperature and pH compared to proteins isolated from mesophilic organisms which have optimal growth conditions at temperatures ranging from 25-40 °C (Brock, 1978). Studies on the thermostability of several AKs from hyperthermophiles have been published and show that these enzymes can survive temperatures of above 80 °C (Ferber *et al.*, 1997; Kath, Schmid

& Schafer 1993; Okajima *et al.*, 2002; Vieille *et al.*, 2003). Thermophiles, including hyperthermophiles can be bacterial or archaeal in origin and therefore the aim was to screen AKs isolated from all these categories to increase the potential range of different AKs for evaluation, in order to identify suitable alternatives for use in the final assay format. In addition, it was important that any AKs identified as a result of this screen, were assessed specifically for potential production of recombinant versions to enable large quantities to be produced.

4.2 Materials and Methods

4.2.1 Isolation of affinity purified AKs

A number of different thermophilic microorganisms of bacterial and archaeal origin were available from an in-house thermophile culture collection (CEPR Thermophile Culture Collection) which included samples of biomass for each of the microorganisms, which had been previously grown using defined growth condition, by Mrs Jean Carr (Sharp & Raven, 1997). This enabled isolation of AKs from the microorganisms listed (Table 4-1).

1 g of biomass from each sample was suspended in 9 volumes of 50 mM Tris-HCl, 200 mM NaCl, pH 7.5 and vortexed for 2-3 seconds to disperse the cell paste. Each aliquot was sonicated on ice for 5 minutes (10 cycles, 30 seconds on/ 30 seconds off) using an MSE Soniprep 150 adjusted to an amplitude of approximately 18 microns. The aliquots were centrifuged at 6000 rpm using a centrifuge (Biofuge Primo) fitted with 6 x 50 mL conical tube rotor for 10 minutes and the supernatants recovered. The supernatants were passed through 0.45 μ Minisart filters (sterile) and collected in separate sterile plastic 50 mL conical tubes and stored at 4 °C.

	Organism	Domain	Growth	T _{opt}	pH _{opt}
1	<i>Aeropyrum pernix</i>	Archaeon	Aerobe	95 °C	7.0
2	<i>Alicyclobacillus acidocaldarius</i>	Bacterium	Aerobe	65 °C	3.5
3	<i>Aquifex pyrophilus</i>	Bacterium	Microaerophile	85 °C	6.5
4	<i>Bacillus caldotenax</i> BT1	Bacterium	Aerobe	65 °C	7.0
5	<i>Bacillus species</i> PS3	Bacterium	Aerobe	65 °C	7.0
6	<i>Bacillus stearothermophilus</i> 11057	Bacterium	Aerobe	65 °C	7.0
7	<i>Bacillus stearothermophilus</i> 12001	Bacterium	Aerobe	65 °C	7.0
8	<i>Bacillus thermocatenulatus</i>	Bacterium	Aerobe	65 °C	7.0
9	<i>Clostridium stercorarium</i>	Bacterium	Anaerobe	55 °C	7.0
10	<i>Meiothermus ruber</i>	Bacterium	Aerobe	60 °C	6.5
11	<i>Pyrococcus furiosus</i>	Archaeon	Anaerobe	95 °C	7.5
12	<i>Pyrococcus horikoshii</i>	Archaeon	Anaerobe	95 °C	7.0
13	<i>Pyrococcus woesei</i>	Archaeon	Anaerobe	95 °C	7.0
14	<i>Rhodothermus marinus</i>	Bacterium	Aerobe	70 °C	6.5
15	<i>Sulfolobus acidocaldarius</i> 98-3	Archaeon	Aerobe	75 °C	2.5
16	<i>Sulfolobus shibatae</i> B21	Archaeon	Aerobe	75 °C	2.5
17	<i>Sulfolobus solfataricus</i> P2	Archaeon	Aerobe	75 °C	2.5
18	<i>Thermoanaerobacter ethanolicus</i>	Bacterium	Anaerobe	65 °C	6.0
19	<i>Thermoanaerobacter thermosulfurogenes</i>	Bacterium	Anaerobe	65 °C	6.5
20	<i>Thermobrachium celere</i>	Bacterium	Anaerobe	60 °C	7.0
21	<i>Thermococcus litoralis</i>	Archaeon	Anaerobe	85 °C	6.5
22	<i>Thermus aquaticus</i> YT1	Bacterium	Aerobe	70 °C	8.0
23	<i>Thermus caldophilus</i> GK24	Bacterium	Aerobe	70 °C	8.0

Table 4-1 Thermophilic microorganisms

Thermostable AKs with inclusion of whether of bacterial or archaeal domain and details of optimal growth temperature and pH of the microorganisms screened for thermostable AKs.

Clarified cell extracts were passed through 1 mL Hitrap Blue affinity columns (Amersham Pharmacia Biotech). The columns were prepared for use by removing the stoppers and connecting 10 mL syringes via the adaptors provided. Care was taken to avoid introducing air to the column at all times. The column preservative buffer was displaced by passing 10 column volumes of binding buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.5) through each column. Following equilibration with binding buffer, supernatants were loaded manually at ~ 0.5-1 mL.min⁻¹. The columns were then washed with 10 further column volumes of binding buffer to elute all non-bound material. Adenylate kinases were affinity eluted using a buffer comprising 10 mM ATP, 10 mM AMP, 10 mM MgCl₂, 50 mM Tris-HCl, 200 mM NaCl, pH 7.5 and collected as 1 mL aliquots and stored at 4 °C until required. The

columns were regenerated by passage of 10 column volumes of 2 M NaCl, followed by a further 10 column volumes of binding buffer.

4.2.2 AK activity assay

All fractions were diluted 1:500 in 50 mM Tris-HCl, pH 7.5 and 100 µl of each dilution was added to a well of a thermostable white microtitre plate (Nunc®) in duplicate. 5 µl of apyrase (Celsis) was added to each well, including controls which comprised; sample (pre-affinity purification); flow through sample; wash stage sample and elution buffer alone. The apyrase was incubated with the samples for 30 minutes at 37 °C and subsequently inactivated by incubation at 65 °C for 10 minutes. The plate was cooled to room temperature and 100 µl of 0.13 mM ADP was added in 15 mM MgAC, 1 mM EDTA, pH 6.7 buffer and incubated for 20 minutes at 70 °C (or 80 or 90 °C for characterisation of thermostability profile) and subsequently cooled to room temperature before the addition of 10 µl of luciferin–luciferase reagent. The relative light units (RLUs) values were read by luminometer (Berthold Orion). For purification, all fractions identified as containing AK activity were pooled and dialysed extensively against PBS or 50 mM Tris-HCl, pH 7.5.

4.2.3 Protein estimation

An estimate of the protein content of the purified AK was performed using 3 methods

- The use of BCA kit (Pierce), according to manufacturer's instruction by comparison to a prepared standard curve

- Absorbance (280nm) using a quartz cuvette and UV spectrophotometer. The concentration of AK was determined by absorption at 280 nm and extinction coefficient for AK defined as $A_{1 \text{ mg/mL}} = 0.52$.
- The use of Biorad DC (detergent compatible) protein assay, according to manufacturer's instructions by comparison to a prepared standard curve

4.2.4 Isolation of genomic DNA

Seven thermophilic organisms were grown using individually defined growth conditions. The procedures for the growth of these organisms are complex and vary according to the origin of the organisms. The fermentation conditions for all the organisms have been described. The organisms were freshly grown using defined growth conditions (Sharp, Raven 1997) by Mrs Jean Carr and Mr Clive Buswell.

Thermophile	Accession number	Database
<i>Aeropyrum pernix</i>	NC 000854	Genbank
<i>Archaeoglobus fulgidus</i>	NC 000917	Genbank
<i>Pyrococcus abyssi</i>	NC 000868	Genbank
<i>Pyrococcus horikoshii</i>	NC 000961	Genbank
<i>Pyrococcus horikoshii</i>	NC 000961	Genbank
<i>Thermotoga maritima</i>	NC 000853	Genbank
<i>Sulfolobus acidocaldarius</i>	NC 007181	Genbank
<i>Sulfolobus solfataricus</i>	NC 002754	Genbank

Table 4-2 List of thermophiles cultured for isolation of genomic DNA

The thermophilic organisms were cultured using defined growth conditions and genomic DNA was isolated to enable potential production of recombinant versions. N.B. *Pyrococcus horikoshii* had two AK genes within

the sequence for cloning. Genomic DNA was isolated from the microorganisms using the Wizard® Genomic DNA Purification Kit (Promega) for Gram positive and Gram negative organisms according to manufacturer's instructions.

4.2.5 PCR amplification of AK using designed primers

The primers were specifically designed based on the genomic sequence of each organism and included restriction enzyme sites for BamH1 or SalI for forward and reverse primers respectively + "GC".

1. *Aeropyrum pernix*

AEROPERNBAM.FOR (25 mer) GCG GAT CCG TGA AGG TGA GAC ACC C

AEROPERNSAL.REV (26 mer) GCG TCG ACT TAG AGG TTC TTT ATG AG

2. *Archaeoglobus fulgidus*

ARCHAEFULBAM.FOR (27 mer) GCG GAT CCA TGA ACC TGA TTT TTC TCG

ARCHAEFULSAL.REV (27 mer) GCG TCG ACT CAG GAT TTG ATC TTT TCC

3. *Pyrococcus abyssi*

PYROABYSBAM.FOR (29 mer) GCG GAT CCA TGA ACA TAC TTA TTT TTG GG

PYROABYSAL.REV (27 mer) GCG TCG ACT CAT CGC CTA TTT TCC TGG

4. *Pyrococcus horikoshii* (AK1)

PYROHORIAK1BAM.FOR (27 mer) GCG GAT CCA TGA ATA TCC TAA TCT TTG

PYROHORIAK1SAL.REV (25 mer) GCG TCG ACT CAT CGC CTA CTC CTA G

5. *Pyrococcus horikoshii* (AK2)

PYROHORIAK2BAM.FOR (32 mer) GCG GAT CCA TGC CCT TTG TAG TCA TTA TTA CG

PYROHORIAK2SAL.REV (28 mer) GCG TCG ACT CAA GCA TAT TCC TTC ACC G

6. *Thermotoga maritima*

THERMMARIBAM.FOR (30 mer) GCG GAT CCA TGA TGG CGT ACC TTG TCT TTC

THERMMARISAL.REV (33 mer) GCG TCG ACT CAT TTA TCA CTC CAC CCT ATT ATC

7. *Sulfolobus acidocaldarius*

SULFACIDOBAM.FOR (30 mer) GCG GAT CCA TGA AGA TTG GTA TTG TAA CTG

SULFACIDOSAL.REV (30 mer) GCG TCG ACT TAC TTC ATA GAC CTT ATT ATC

8. *Sulfolobus solfataricus*

SULFSOLBAM.FOR (30 mer) GCG GAT CCA TGA AAA TAG GTA TAG TAA CTG

SULFSOLSAL.REV (32 mer) GCG TCG ACT CAC ATT AAG GAA TTT ATT ATT TC

10 µL of Thermopol buffer (20mM Tris-HCl, 10mM NH₄ SO₄, 10mM KCl, 2mM MgSO₄, 0.1% Triton X-100, pH 8.8) (New England Biolabs) 2.5 µL (2mM) MgCl₂, 1 µL dNTPs (200 µM), 1 µL of each primer (forward and reverse) diluted to 100 pmoles, 1 µL of genomic DNA template, 1 µL of Vent polymerase (New England Biolabs) plus 82.5 µL of distilled H₂O were added to 0.2 mL PCR tubes. The negative control consisted of the DNA template replaced by distilled H₂O. A standard PCR programme was run overnight. The PCR products were analysed by running on a 1.2 % pre-cast agarose gel (E-Gel®, Invitrogen) with a 100 bp and 1 kb DNA ladders (Invitrogen). The relevant bands were excised from the gel and purified using Qiaquick Gel Extraction kit (Qiagen), according to the manufacturer's instructions.

4.2.6 TOPO Cloning and Transformation

An A-overhang was created on the gel extracted PCR products to allow cloning into TOPO TA vector (Invitrogen). This was achieved by the addition of 8 µL of PCR products with 1 µL of Taq polymerase and 1 µL of 10 mM dATP and incubation at 72 °C for 10 minutes. Following this, the TOPO cloning and transformation reaction was carried out according to manufacturer's instructions. Once the procedure was complete, 10 µL or 100 µL of each transformation reaction was spread on an L-agar plate containing 100 µg.mL⁻¹ ampicillin. The plates were incubated overnight at 37 °C and analysed for colony growth the next day.

4.2.7 Sub-cloning of AK gene into pET28a

DNA was isolated from sub-clones using Wizard® SV 96 Plasmid DNA Purification (Promega) following manufacturer's instructions. The correct insert was confirmed by performing a BamHI / SalI restriction enzyme digest prior to sequencing by the addition of 8 µL of plasmid preparation, 1 µL of BamHI, 1 µL of SalI, (Restriction enzymes, New England Biolabs) 2 µL of Sal buffer and 8 µL of distilled H₂O to a microcentrifuge tube followed by incubation for 3 hours at 37 °C. The results were analysed by running on a 1.2 % agarose E-gel (E-Gel®, Invitrogen) and visualising with UV light using the Bio-Rad Gel Documentation system. The BamHI / SalI digest fragment were excised from the gel and purified using Qiaquick Gel Extraction kit (Qiagen), according to the manufacturer's instructions. The pET 28a vector (Novogen) was BamHI and SalI digested and dephosphorylated using 1 unit of alkaline phosphatase for 30 minutes at room temperature. Ligation of the Bam / Sal fragment was carried out by the addition of 3 µL of the vector, 7 µL of the insert, 3 µL of 5 x ligase buffer, 1 µL of 10 mM ATP and 1 µL (0.2-0.4U / µL) of the DNA ligase and incubating overnight at 16 °C. After overnight incubation, a further 0.5 µL of T4 DNA ligase was added to each reaction to ensure full ligation and incubated for 20-30 minutes at room temperature. 2 µL of the ligation mix was added to an aliquot of *Escherichia coli* (*E. coli*) JM109 cells (Stratagene) and incubated on ice for 5 minutes. Cells were heat shocked at 42 °C for 1 minute and returned to ice. 250 µL of SOC medium was added and further incubated for 1 hour at 37 °C, while shaking horizontally. 10 and 100 µL aliquots were inoculated onto L-agar plates containing kanamycin (35 µg.mL⁻¹). The agar plates were incubated at 37 °C overnight and subsequently analysed for colony growth. Colonies (3) were picked and inoculated into 5mL of L-Broth containing kanamycin (35 µg.mL⁻¹) and incubated overnight at 37 °C.

4.2.8 Transformation of *Sulfolobus* AK into *E. coli*

Sulfolobus acidocaldarius adenylate kinase in the vector pET3a (Amp^R) was supplied by Professor G Schafer (Vonnheim *et al.*, 1998) and *Sulfolobus acidocaldarius* adenylate kinase in the vector pET28a were transformed into BL21 (DE3) (Novagen) or JM109 (DE3) *E. coli* competent cells (Promega). In addition codon enhanced strains of *E. coli* BL21 (DE3) pACYC ILE ARG 10 cells, expressing rare tRNAs suitable for enhancing expression levels of AT-rich genes were used. (Novogen) (Zdanovsky & Zdanovskaia, 2000). 20 µL aliquot of the appropriate cells were transformed by the addition of 2-4 µl of plasmid DNA, the cells were left on ice for 30 minutes and then heat shocked for 50 seconds at 42 °C. The cells were returned to ice for a further 2 minutes followed by the addition of 1 mL of L-Broth and incubated at 37 °C for 1 hour in a shaking incubator. The cells were subsequently plated on L-agar plates containing antibiotics ampicillin, (100 µg.mL⁻¹); or kanamycin (25 µg.mL⁻¹) plus chloramphenicol (35 µg.mL⁻¹) 250 µl per plate, as appropriate, and incubated overnight at 37 °C. The cells transformed with the AK genes were grown in 8 L fermenters and cell paste harvested (CEPR Fermentation Plant).

4.2.9 Mini-expression study

Three colonies were picked from each plate and inoculated into 3 mL of L-broth containing kanamycin (25 µg.mL⁻¹) and chloramphenicol (35 µg.mL⁻¹). The cultures were incubated at 37 °C for 3.5 hours, while shaking at 200 rpm. The cultures were induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) (500 µM) and grown for a further 2 hours at 37 °C. The cells were pelleted by centrifugation at 13,500 rpm (19150 x g) in microcentrifuge for 10 minutes. The supernatant was discarded and the pellet resuspended in 300 µl of PBS and sonicated by 3 cycles of 30 seconds on / 30 seconds off at

approximately 18 μ at full power on ice. The resulting solution was centrifuged at 13,500 rpm for a further 5 minutes and the supernatant retained for analysis.

4.2.10 Calculation of specific activity

Specific activity is defined as $\mu\text{mol of ATP mg}^{-1} \cdot \text{min}^{-1}$.

1 unit of AK \approx 1 μmol of product formed per minute at a given temperature i.e. 1 μmol of ATP generated from ADP per minute (BioThema AB, ATP kit SL 144-041). Purified AK sample was diluted 1:200,000 and 1:400,000 in 15 mM magnesium acetate buffer + 1 mM EDTA, pH 6.7. ADP was prepared as a 135 μM solution in the above buffer and 160 μL was added to appropriate wells. Dilutions of AK were added to the wells (1:100, 2 μL / well). The assay was controlled by the inclusion of wells excluding ADP and AK respectively. The microtitre plate was incubated at 70 $^{\circ}\text{C}$ for 30 minutes then cooled to 20 $^{\circ}\text{C}$ using the Thermocycler (Techne). 30 μL of the ATP reagent (Luciferin-luciferase) (BioThema) was added to each well and the RLU measured in a plate luminometer (Berthold Orion). ATP standard (supplied) was added to the wells (10 μL) and ATP produced was calculated using the equation: $-\text{ATP}_{\text{smp}} = 10^{-7} \times I_{\text{smp}} / (I_{\text{smp+std}} - I_{\text{smp}})$.

4.2.11 Heat treatment purification step

Approximately 10 g of frozen cell paste was weighed and re-suspended in 3 volumes of 20 mM Tris-HCl, 1 mM EDTA containing 900 mM NaCl at pH 7.5. The cells were sonicated (MSE Soniprep 150) on ice (~20 microns) using 25 cycles of 30 seconds on/ 30 seconds off and the supernatant collected by centrifugation at 6000 x g for 30 min at 4 $^{\circ}\text{C}$. Heat

labile proteins were removed by heat treatment at 80 °C in a water bath for 20 min and the resulting precipitate removed by centrifugation at 6000 rpm for 30 min.

4.2.12 Affinity purification

HiTrap Blue columns 3 x 5 mL (Amersham Pharmacia) were joined in sequence and equilibrated with 5 column volumes of 20 mM Tris-HCl, 1 mM EDTA, 900 mM NaCl, pH 7.5 using FPLC chromatography (Pharmacia) 150 mL of the heat-treated supernatant was applied to the column using a Superloop (GE Life Sciences) and the flow through collected. After loading of the sample, the column was washed with 20 mM Tris HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5 at a rate of 0.2 mL.min overnight and a sample of wash solution collected. The column was re-equilibrated with a further 5 column volumes of 200 mM Tris-HCl, 1 mM EDTA, 900 mM NaCl, pH 7.5 and the AK eluted using buffer containing 10 mM ATP, 10 mM AMP, 10 mM MgCl₂ in 200 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5 at a rate of 1 mL.min and 2.5 mL fractions collected. Regeneration of the column was achieved by washing with further 5 column volumes of elution buffer followed by 5 column volumes of 200 mM Tris-HCl, 1 mM EDTA, 900 mM NaCl, pH 7.5 and then rinsed with 20 % ethanol.

4.2.13 Gel filtration

A Superdex 75 Hiload 16/60 preparative column was pre-equilibrated with 50 mM Tris-HCl, pH 7.5 with approximately 250 mL of buffer using FPLC chromatography (Pharmacia). A 2 mL sample of affinity purified, heat treated supernatant was loaded via a sample loop and the buffer re-applied to the column. The peak was identified by measuring

absorbance (280nm). Fractions were collected in 2.5mL aliquots and the AK activity assessed by AK activity assay.

4.2.14 SDS-PAGE assessment of purification of AK

Proteins were analysed by SDS-PAGE on 4-12 % Bis Tris gels (Invitrogen NuPAGE® Novex Gels). Samples were prepared by adding each sample (25 µL) to 75 µL of distilled H₂O. NuPAGE LDS (x 4) sample buffer (25 µL) was added to each and the samples heated to 99 °C for 2 minutes. Novex Mark 12 Standards were used as markers and the gel apparatus was set up per manufacturer's instructions. 5 µL of markers or 25 µL of samples were added to relevant wells and the gel run for 50 minutes in MOPS running buffer (Invitrogen) and proteins visualised using the Colloidal Blue staining kit (Invitrogen) according to the manufacturer's instructions. The gel was photographed using the Biorad gel documentation system.

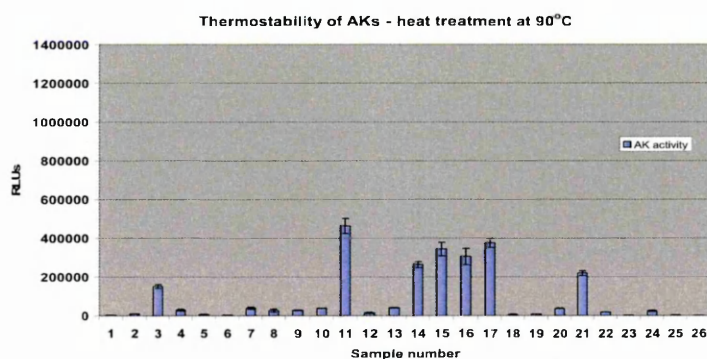
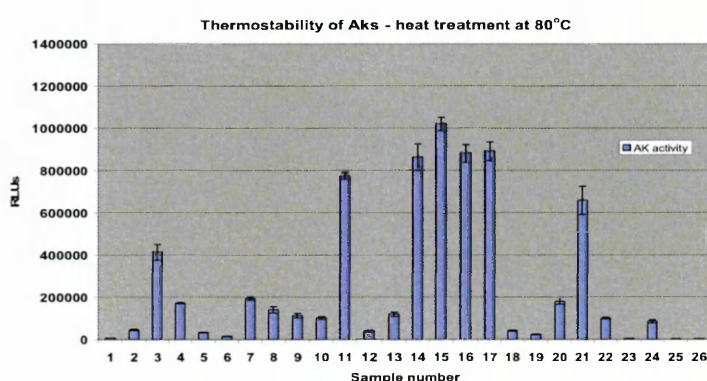
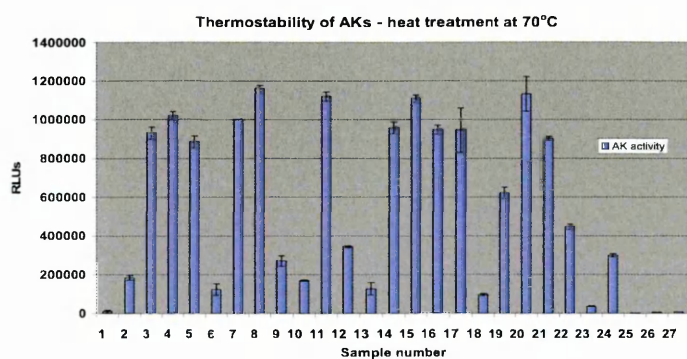
4.2.15 Characterisation of dynamic range of AK

Sulfolobus acidocaldarius AK was diluted over a log range in 50 mM Tris-HCl, pH 7.5 and 100 µL of each dilution was added to a well of a thermostable white microtitre plate (Thermofast, Abgene) in duplicate. 100 µL of 0.13 mM ADP was added in 15 mM MgAC, 1 mM EDTA, pH 6.7 buffer and heated to 30, 50 and 70 °C for 20 minutes then cooled to room temperature before the addition of 30 µL of luciferin–luciferase reagent (Biothema) to each well. The RLU values were read by luminometer.

4.3 Results

4.3.1 Screening for alternative thermostable adenylate kinases

The aim of this work was to identify potential candidate AKs with improved thermostable properties, for comparison to the AK isolated from *Bacillus stearothermophilus* used previously in the model assay. This was achieved through access to a thermophile culture collection (CEPR Thermophile Culture Collection), which enabled isolation of AKs from a range of thermophilic organisms, to determine if any could be identified which were optimal for the purpose of further development of the ultra-sensitive assay. In order to set up a screen of the thermophiles, biomass was prepared from each of the organisms on the list (Table 4-1) and a method of isolating AKs from this biomass established (4.2.1). This enabled a large number of AKs to be compared from a range of bacterial and archaeal thermophiles with different inherent properties. The initial screening of twenty-four thermophilic microorganisms yielded a number of organisms with highly active and thermostable AKs and the results are shown in Figure 4-1. An assessment was carried out at increasing temperatures of 70, 80 and 90 °C to fully evaluate the thermostability of the different AKs isolated from a range of microorganisms.



Key – Sample number

1. *Aeropyrum pernix*
2. *Alicyclobacillus acidocaldarius*
3. *Aquifex pyrophilus*
4. *Bacillus caldodenax* BT1
5. *Bacillus species* PS3
6. *Bacillus stearothermophilus* 11057
7. *Bacillus stearothermophilus* 12001
8. *Bacillus thermocatenuatus*
9. *Clostridium stercorarium*
10. *Meiothermus ruber*
11. *Pyrococcus furiosus*
12. *Pyrococcus horikoshii*
13. *Pyrococcus woesei*
14. *Rhodothermus marinus*
15. *Sulfolobus acidocaldarius* 98-3
16. *Sulfolobus shibatae* B12
17. *Sulfolobus solfataricus*
18. *Thermoanaerobacter ethanolicus*
19. *Thermoanaerobacter thermosulfurogenes*
20. *Thermobrachium celere*
21. *Thermococcus litoralis*
22. *Thermus aquaticus* YT1
23. *Thermus caldophilus* GK24
24. *Thermus thermophilus* HB8
25. control 1
26. control 2
27. control 3

Figure 4-1 Assessment of the thermostability at 70, 80 and 90 °C of AKs

The AKs were isolated from the biomass by affinity chromatography using selective absorption and desorption from Cibacron Blue 3A (Blue Sepharose). The supernatant was treated with apyrase to destroy ATP present from elution buffer and apyrase subsequently inactivated by heat treatment at 65 °C prior to AK assay.

In Figure 4-1, many AKs showed little or no loss of activity at 80 °C compared to a base line of 70 °C. In addition, some of the identified AKs retained up to 30% of their activity at 90 °C. This result demonstrated that potentially more suitable candidate AKs than *Bacillus stearothermophilus* AK were available for inclusion in an assay format utilising heat treatments of up to 80 °C to remove background signal.

The AKs identified from this round of screening with suitable thermostable properties for use in the assay were *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Sulfolobus solfataricus*, *Sulfolobus shibatae* B21, *Rhodothermus marinus* and *Thermococcus litoralis*, which all demonstrated considerable residual activity of ~ 30 % following incubation at 90 °C and high activity at 80 °C. This identified them as potentially useful for the developed assay to enable inclusion of heat steps of temperatures of 70-80 °C, to remove any residual contaminating thermolabile AK present in a large excess of biological material.

4.3.2 Production of recombinant thermostable AKs

The results of the screening showed that there were several AKs with the required properties for use as an enzyme label in the proposed assay. In order for sufficient quantities of AK to be obtained, which would enable purification and subsequent conjugation to antibody, it was important that a method was developed to enable production of a stock of enzyme of consistent quality. It was therefore decided that recombinant DNA technology would provide the best approach. A difficulty was encountered however, as it was discovered that there were few known genomic sequences to enable design of specific primers for PCR amplification of AKs. On further investigation, it was found that seven of the thermophiles included in the initial screening

had known genomic DNA sequences with identified AK genes. These were *Aeropyrum pernix*, *Archaeoglobus fulgidus*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Thermotoga maritima*, *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* and the latter two were identified as highly thermostable from the screening process. It was therefore decided that recombinant AKs would be produced of these 6 thermophiles. This would enable assessment and comparison of the AKs from two identified as being highly stable at temperatures of 80 °C and above with AKs from alternative sources. It was decided that it was worth assessing AKs with perhaps less thermostability but with other potentially suitable qualities such as successful over-expression in *E. coli* for reasons of practicality.

In order to carry out PCR amplification of the AK genes, genomic DNA had to be isolated from biomass obtained by growth of the thermophiles using the individually defined growth conditions (4.2.4). A method of isolating genomic DNA from Gram positive microorganisms was originally attempted for all the cell pastes. However it was found that this method was only successful for *Thermatoga maritima*, which was the only bacterial culture and also known to be a Gram positive organism. Therefore an alternative method of genomic DNA extraction had to be identified for the archaeons. A method suitable for extraction of genomic DNA from Gram negative microorganisms was then trialled and found to be successful (Wizard® Genomic DNA Purification Kit). The genomic DNA extracted from all the thermophiles was used as templates for PCR amplification of the adenylate kinase genes from each organism (4.2.5) and analysis of the PCR product carried out using a 1% agarose gel (E-gel) to identify whether any of the PCR products were of the expected size of ~600 bp.

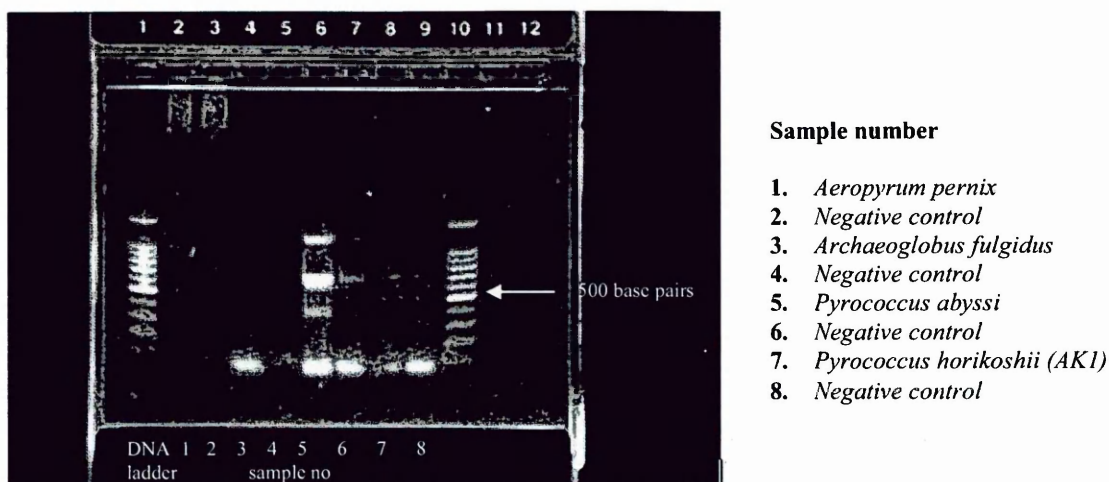


Figure 4-2 Analysis of PCR products

Genomic DNA was extracted from biomass for all the microorganisms in the above list. The DNA was used as template for the PCR amplification of the AK genes using specially designed primers based on the known DNA sequence of the microorganisms. Negative controls were included between each sample lane, with water replacing genomic DNA template

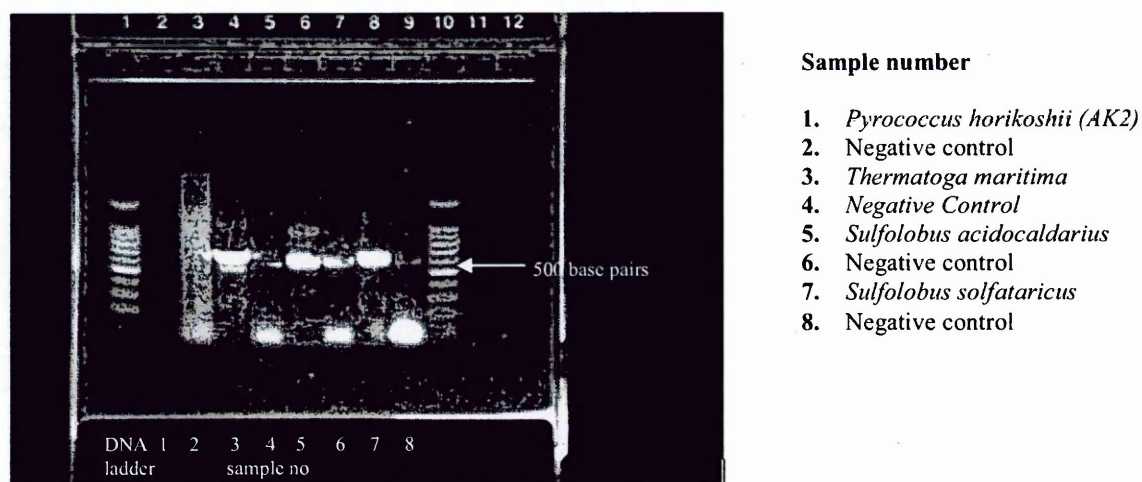


Figure 4-3 Analysis of PCR products

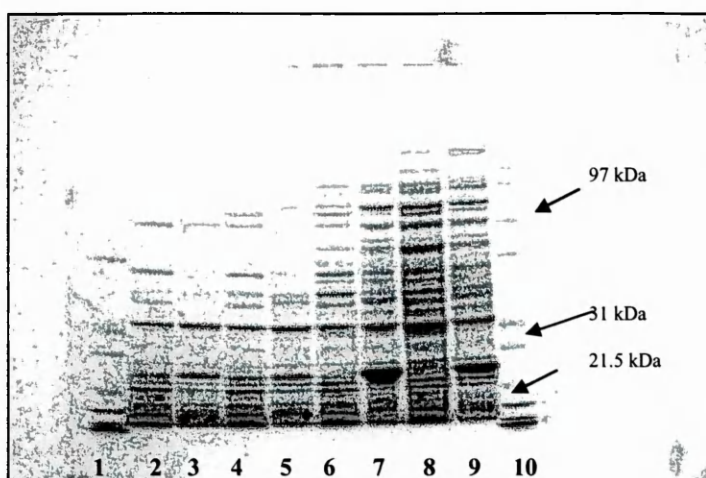
The expected size of the PCR products was ~ 600 base pairs and the results in Figure 4-2 and Figure 4-3 indicate that AK genes had been successfully PCR amplified from four of the samples. The samples were *Pyrococcus abyssi*, *Thermatoga maritima*, *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*. Production of recombinant AKs was continued

by cloning of the PCR products into a TOPO TA vector (4.2.6) before subsequent sub-cloning into a pET28a expression vector (4.2.7). In addition, a sample of recombinant *Sulfolobus acidocaldarius* AK contained within the vector pET3a was kindly donated by Professor G Schaeffer (Vonnheim *et al.*, 1998) and enabled a stock of AK to be prepared initially while work was carried out to produce a more suitable recombinant AK.

4.3.3 Over-expression of recombinant AKs

In order to determine the success of the production of recombinant AKs within the vector pET28a expression studies were performed as described in section 4.2.9.

In addition, over-expression of *Sulfolobus acidocaldarius* AK which had been supplied within pET3a vector (Vonnheim *et al.*, 1998) was carried out by transformation into *E. coli* BL21 (DE3) cells and this stock of recombinant AK was purified as described previously (4.2.11). The yield obtained on over-expression was rather low (0.5-1 mg.L⁻¹), however it enabled work to begin on various stages of assay development while production of an in-house recombinant AK was developed with a higher yield.

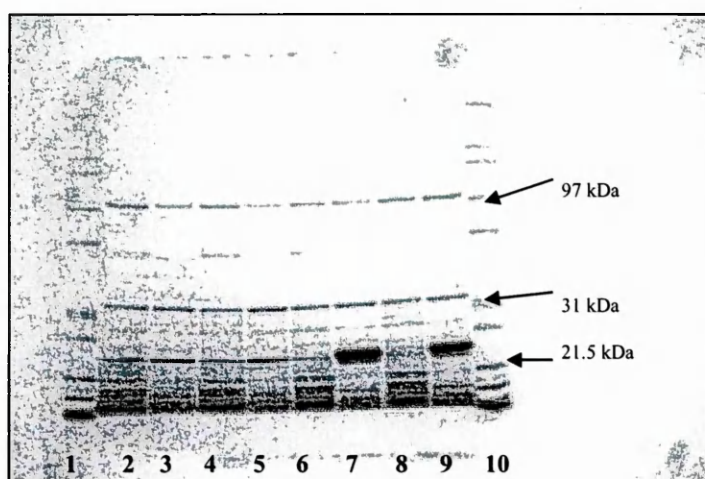


Key

1. Molecular weight markers
2. *Pyrococcus abyssi* (pre induced sample)
3. *Pyrococcus abyssi* (post induced sample)
4. *Thermotoga maritima* (pre induced)
5. *Thermotoga maritima* (post induced)
6. *Sulfolobus acidocaldarius* (pre - induced)
7. *Sulfolobus acidocaldarius* (post induced)
8. *Sulfolobus solfataricus* (pre)
9. *Sulfolobus solfataricus* (post-induced)
10. Molecular weight markers

Figure 4-4 SDS-PAGE analysis of over-expressed recombinant AKs

Over-expressed AKs from *Pyrococcus abyssi*, *Thermotoga maritima*, *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* pre and post induction (see key). Supernatant containing the over-expressed protein was analysed by SDS-PAGE on 4-12 % Bis Tris gels with MOPs running buffer. The proteins on the gel were visualised using Colloidal Blue Staining kit (Invitrogen) and imaged by Biorad gel documentation system.



Key

1. Molecular weight markers
2. *Pyrococcus abyssi* (pre induced sample)
3. *Pyrococcus abyssi* (post induced sample)
4. *Thermotoga maritima* (pre induced)
5. *Thermotoga maritima* (post induced)
6. *Sulfolobus acidocaldarius* (pre - induced)
7. *Sulfolobus acidocaldarius* (post-induced)
8. *Sulfolobus solfataricus* (pre)
9. *Sulfolobus solfataricus* (post-induced)
10. Molecular weight markers

Figure 4-5 SDS-PAGE analysis of over-expressed recombinant AKs (heat treated)

Pyrococcus abyssi, *Thermotoga maritima*, *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* pre and post induction (see key).

The supernatant containing the over-expressed protein was heat treated by incubation at 80 °C for 20 minutes before centrifugation to remove the heat labile proteins from the *E. coli* host, prior to being re-analysed by SDS-PAGE 4-12 % Bis Tris gels with MOPs running buffer. The proteins were visualised using Colloidal Blue Staining kit (Invitrogen) and the gels by Biorad gel documentation system.

SDS-PAGE analysis demonstrated that only recombinant AK from *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* had been successfully over-expressed and also retained thermostability as shown by the presence of protein bands of approximately 24 kDa in both the pre and post heat treated samples (Figure 4-4 & Figure 4-5).

The results of the SDS-PAGE analysis showed that the recombinant AK derived from *Sulfolobus acidocaldarius* had been over-expressed in a somewhat greater quantity within the same volume of sample, denoted by the presence of a larger band of the expected molecular mass (~24 kDa). It was therefore decided that work would focus on the production of this recombinant AK as this would enable production of sufficient quantities to enable preparation of a large batch of AK for use in conjugations to anti-PrP antibodies. The yield of this recombinant AK was substantially greater than found with *Sulfolobus acidocaldarius* recombinant AK within pET 3a vector and was shown to be in the region of 5-10 mg.mL⁻¹

4.3.4 Purification of recombinant AK

The purification of the *Sulfolobus acidocaldarius* recombinant AK was achieved in a two stage process (4.2.11). The initial stage of work on over-expression of recombinant AKs analysed by SDS-PAGE showed the effect of heat treatment at 80° C. This demonstrated

the efficacy of this as a first stage of purification which inactivated and precipitated proteins derived from the host *E. coli*. Figure 4-4 & Figure 4-5 (Lanes 7), show *Sulfolobus acidocaldarius* recombinant AK with and without the inclusion of a heat step of 80 °C for 20 minutes which precipitates heat labile proteins within the supernatant. SDS-PAGE analysis demonstrates the effectiveness of this first stage purification with removal of approximately 80% of the heat labile, *E. coli* derived protein.

Additional work took place to develop a method of further purification and this was achieved by an affinity purification step involving adsorption of the enzyme to a Blue Sepharose column, followed by specific elution with a low concentration of adenylate kinase co-factors (AMP+ATP in the presence of magnesium ions) (4.2.11). This was followed by evaluation of the additional benefit of inclusion of a gel filtration step using a preparation grade Superdex column. (4.2.13). The results of a comparison of the addition of sequential purification steps of heat treatment; affinity purification and gel filtration are shown in Figure 4-6. The results of the SDS-PAGE analysis show that there was no additional benefit from the inclusion of this step and in fact the protein band is purified to near homogeneity after the first two steps and the inclusion of gel filtration results in a loss of sample. It was therefore decided that this step would be omitted and purification of bulk quantities of *Sulfolobus acidocaldarius* AK would be achieved by the use of heat step followed by affinity purification alone.

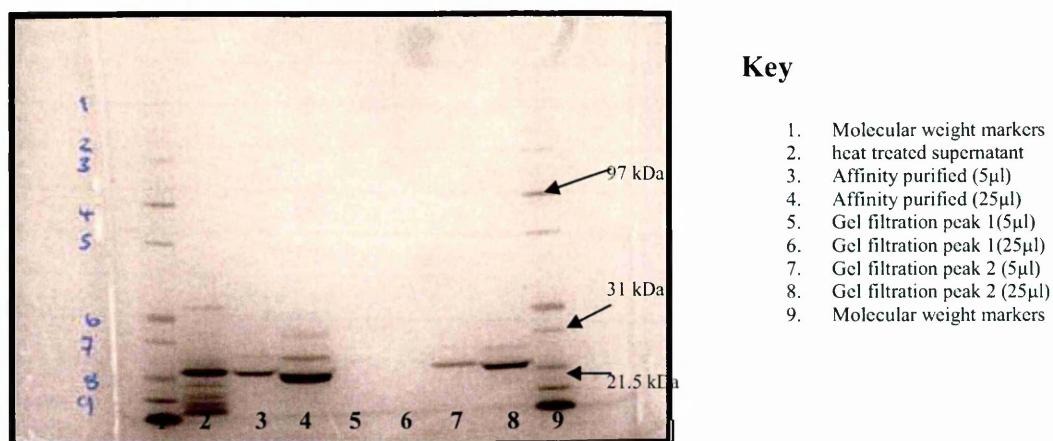


Figure 4-6 SDS-PAGE analysis of comparison of *Sulfolobus acidocaldarius* AK

Analysis of *Sulfolobus acidocaldarius* AK purified by three different methods; lane 2; heat treatment alone, lanes 3-4; affinity purification following heat treatment; lanes 5-8; heat treatment / affinity purification followed by gel filtration. Proteins were analysed by SDS-PAGE on 4-12% Bis Tris gels with MOPs running buffer. The proteins were visualised using Colloidal Blue Staining kit (Invitrogen) and the gel using Biorad gel documentation system.

4.3.5 Calculation of Specific Activity

The specific activity of the purified batch of *Sulfolobus acidocaldarius* AK was calculated as 2000 units.mg⁻¹. One unit of specific activity is defined as that producing 1 µmol of ATP.min⁻¹ at 70 °C (4.2.10).

4.3.6 Characterisation of dynamic range of AK

In order to further characterise *Sulfolobus acidocaldarius* AK and its thermostable properties, it was decided to investigate the results of incubation with ADP at varying temperatures, to assess the effect on the production of ATP by this AK (Figure 4-7).

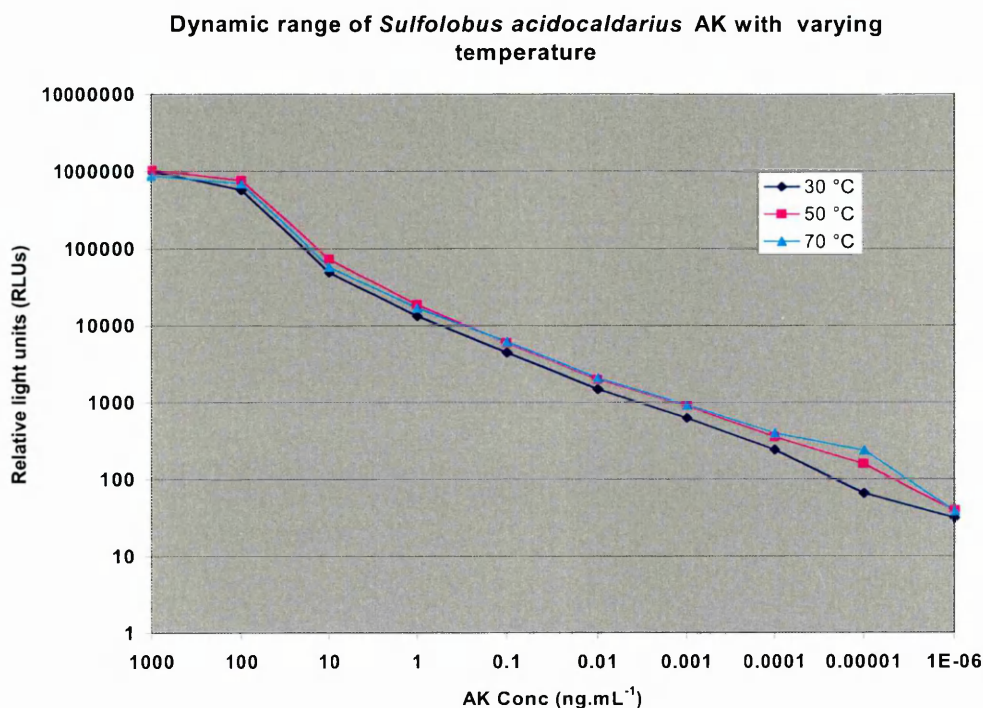


Figure 4-7 Characterisation of the dynamic range of AK over varying temperatures

A standard AK assay was carried out by titrating AK in ten-fold dilutions over a long log range and incubation with ADP was carried out at varying temperatures of 30, 50 and 70 °C, before cooling to room temperature and addition of L/L reagent. RLU values read on plate luminometer.

The result showed that the enzyme had an exceptionally long dynamic range of greater than 8 logs, which was unaffected by temperatures. This result also showed that there was a theoretical limit of detection of AK of less than 100 attograms.

4.3.7 Long-term storage of purified thermostable AKs

Freeze-drying was evaluated as a method for the long-term preservation and storage of purified thermostable adenylate kinase. This was assessed using a short time course trial which was achieved by freeze-drying samples of two different batches of purified enzyme.

These samples were then reconstituted with water and the adenylate kinase activity of pre and post freeze-drying samples was calculated by a standard bioluminescent assay. There was an approximate 15 % loss of activity in both cases (results not shown), which was considered acceptable and therefore this method was considered suitable for use in the preparation of stocks of purified enzyme. Long term storage of adenylate kinase by this method however, has still to be evaluated.

4.4 Discussion

The results discussed in Chapter 3, established that successful preparation of *Bacillus stearothermophilus* AK antibody conjugates was achievable and that utilisation of the inherent properties of thermostability was possible to reduce background signal from other s of AK. It was identified however, that although the *Bacillus stearothermophilus* AK was suitable for heat steps of temperatures up to 60 °C, there was a noticeable loss of activity at 70 °C. As the ultimate objective of the developed assay was to enable detection of potentially very low amounts of infectious prion material within a possible vast over-excess of biological material such as neuronal tissue, it was imperative that the enzyme label was able to retain activity at temperatures sufficiently high to inactivate heat labile AKs and to enable sensitive detection in this format. In addition, the cost of purchasing a commercial AK was financially prohibitive especially to allow all the stages of the assay to be fully developed and optimised. Therefore an alternative AK was necessary, which would exhibit the robust thermostable qualities required and which could be economically produced in a recombinant form to enable production and purification of sufficient quantities to develop this assay format fully.

During this time, a sample of plasmid DNA containing recombinant *Sulfolobus acidocaldarius* AK in the vector pET3a was kindly donated by Professor G Schaeffer (Vonnheim *et al.*, 1998). This was transformed into *E. coli* (BL21) cells and subsequently over-expressed and purified to provide an initial stock of AK for use in initial development. However, the use of this vector was not ideal as induction of over-expression of recombinant protein is not well controlled and the overall quantity of recombinant protein produced using this system, tends to be low. The results of initial work on over-expression showed that this was the case with this recombinant thermostable *Sulfolobus acidocaldarius* AK and the yield was approximately 0.5 mg per Litre of culture. Therefore work continued to identify potentially alternative AKs with perhaps more suitable qualities for use and subsequent development of a more successful recombinant version.

The screening of biomass from twenty four thermophilic microorganisms resulted in the identification of a number of highly active and thermostable AKs, showing no appreciable loss of activity at temperatures of up to 80 °C and indeed, a small number retained enzyme activity at temperatures of 90 °C. Hence it was clear that there were several potential alternative AKs with superior qualities for use in the development of this assay format. Once candidate AKs had been identified the work then focussed on production of recombinant versions; however the available information on the genomic sequence of the identified thermophiles and AK genes was sparse. However, several thermophiles had published sequences of AK genes two of which were candidate AKs identified during the screening for thermostability. This enabled specific primers to be designed for these plus a number of other thermophilic AKs for comparison (Bae, Phillips 2004; Glaser *et al.*, 1992; Kath, Schmid & Schafer 1993; Okajima *et al.*, 2002). The thermophiles with known sequences were *Pyrococcus abyssi*, *Thermatoga maritima*, *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*.

Work therefore concentrated on attempts to produce these four AKs recombinantly. It had been previously published that there were difficulties associated with efficient over-expression of recombinant proteins from e.g. microorganisms such as archaeons or Clostridial neurotoxins in traditional host strains such as *E. coli* (Kim *et al.*, 1998; Zdanovsky & Zdanovskaia, 2000). This was thought to be due to the AT rich content of the *Sulfolobus acidocaldarius* and Clostridial neurotoxin genomes being problematic for over-expression in the GC rich *E. coli*. In addition there is codon usage for arginine and isoleucine which are rare in *E. coli*. It had been hypothesised that introduction and amplification of these specific tRNA genes for rarely used codons can vastly improve levels of over-expression of the recombinant proteins in the *E. coli* host strain. Therefore a host strain was procured which expressed the rare tRNAs for use in expression of the recombinant AKs (Zdanovsky & Zdanovskaia, 2000). Work therefore concentrated on transformation into a codon enhanced *E. coli* strain expressing rare tRNAs. This method was shown to be successful with an approximate 10-fold increase in yield to 5-10 mg per Litre of culture.

Attempts to produce recombinant versions of these identified AK were analysed by mini-expression studies followed by analysis by SDS-PAGE and included heat treatment of the supernatants at 80 °C. The results showed that the only recombinant AKs produced from *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* had been successful. Further analysis of results of expression studies showed that *Sulfolobus acidocaldarius* was more efficiently over-expressed, therefore it was decided that this AK would become the focus for further development.

A purification process was developed for the recombinant *Sulfolobus acidocaldarius* AK and the initial plan was to develop a three stage purification process in order to achieve maximum purification. Analysis of the first stage of purification however, showed that the

initial heat treatment of 80 °C for 30 minutes was an extremely efficient purification step which effectively removed a large proportion of the contaminating thermolabile proteins from the *E. coli* host strain and achieved approximately 80 % purity. The addition of the affinity purification step using adsorption to a Blue Sepharose column, followed by specific elution with a low concentration of adenylate kinase co-factors (4.3.4) demonstrated purification to near homogeneity on SDS-PAGE analysis, whereas the addition of a gel filtration step merely resulted in loss of sample. It was therefore decided that the addition of a gel filtration step during production of a batch of AK, would provide no additional benefit. It was clear from these results that the inherent thermostable properties of the AK provided a very effective first stage purification method. The fact that only two purification steps were required meant that problems associated with loss of material during the purification process were largely avoided and enabled a preparation of a stock of approximately 100 mg of purified AK for use in development of the assay.

In order to carry out conjugation reactions, it was a requirement for a reliable method of protein estimation to be identified and a number of methods were evaluated. It was found however that the values given by analysis of the same sample by BCA, Biorad DC assay and measurement of absorbance (280 nm) were vastly different. In fact the differences were in the order of 100 fold between the different methods. The reasons for this were never resolved within the period of the project and eventually Biorad DC was used along with SDS-PAGE and a known standard. It was hypothesised that it may have been due to the structure of this AK which has been shown to be trimeric with a highly condensed hydrophobic structure (Vonnheim *et al.*, 1998).

Some further investigation was carried out in order to further characterise the recombinant *Sulfolobus acidocaldarius* AK and the range of activity at different temperatures. The results were surprising as there was an expectation that the AK from a thermophile would

have an optimum activity at temperatures required for growth of the source organism and for *Sulfolobus acidocaldarius* the optimum temperature for growth is 75 °C. The results of the characterisation of the AK activity showed that the enzyme was unaffected at a range of temperatures of 37-70 °C. A linear range of activity was shown over greater than 8 logs and demonstrated that the potential limit of detection of this enzyme was less than 100 attomoles. This demonstrated further the suitability of *Sulfolobus acidocaldarius* AK for use in the development of an ultra-sensitive assay format as the enzyme is clearly robust and likely to be unaffected by all the various assay conditions encountered but with a clear potential to reach very sensitive detection limits.

A method of long term storage of the purified AK had to be established to allow the next stages of assay development to progress and freeze drying was shown to be suitable as it resulted in a fairly minimal loss of approximately 15 % of activity which was considered to be acceptable. The long term storage of the purified freeze dried stock of AKs was not formally evaluated, however it was noted that the stocks of AK stored in this way during the course of this project showed no appreciable loss of activity over a time period of one to two years.

In summary, the results demonstrated that an alternative thermostable AK had been identified and recombinant version successfully produced: a purification method established and a substantial stock of AK of consistent quality prepared. In addition, a method of successful long term storage was identified. The results also demonstrated that this thermostable AK showed surprising versatility and the potential to attain sensitivity of less than 100 attomoles if used in a suitable format.

5 Production of anti-prion polyclonal antisera

5.1 Introduction

It was considered beneficial for the project that antibodies of the desired specificities to PrP were developed. Antibodies produced to recombinant PrP e.g. 6H4 (Prionics) were available commercially but the cost was financially prohibitive, and therefore the aim was the production of an in-house batch of antisera suitable for use in the development of an ultra-sensitive prion ELISA.

One of the biggest challenges in the development of a sensitive and specific detection method for CJD is the nature of the infectious agent itself, which is believed to be an altered form of normal cellular protein, with no discovered nucleic acid, which rules out the use of traditional polymerase chain reaction (PCR) based assays, and this is combined with a lack of host immune response and therefore the use of serology (Prusiner, 1998). It has also been acknowledged that PrP is poorly immunogenic (Prusiner *et al.*, 1993). Considerable difficulties have been encountered in the development of antibodies to the putative disease associated form PrP^{Sc}, because of the misfolded protein conformation therefore resulting in few disease specific epitopes for targeting. Only a few antibodies have been described to date that claim to detect PrP^{Sc} and distinguish it from PrP^C (Korth *et al.*, 1997; Moroncini *et al.*, 2004; Paramithiotis *et al.*, 2003).

A strategy was therefore designed to boost antibody responses to the antigen in order to generate polyclonal sera which would hopefully fulfil this purpose. This involved the design of peptides based on highly conserved regions of mouse, bovine and human PrP (Wopfner *et al.*, 1999). This was combined with a novel method of antigen presentation

and the use of an unusual prime / boost strategy to produce polyclonal sera (De Silva, Egodage & Wilson 1999; van der Werf *et al.*, 1994). The aim was to characterise the produced antisera for reactivity with recombinant PrP, non-infectious mouse brain and infectious mouse brain to determine suitability for use in the development of this assay.

5.2 Materials and Methods

5.2.1 Design of prion derived peptides

Six regions of high homology between mouse, human and bovine prion protein amino acid sequences were selected for peptide synthesis and antibody production (Wopfner *et al.*, 1999). These peptides were synthesised commercially with an N- terminal cysteine residues and with and / or without a C-terminal cysteine and conjugated to mycobacterial purified protein derivative (PPD) carrier protein to effect a more immunogenic antigen presentation. These mixed inocula, including structurally constrained peptides (with cysteine residues at both ends) and linear peptide antigens were used to immunise rabbits following an initial priming stage with Bacille Calmette-Guerin (BCG) (vaccine for intradermal use (Statens Serum Institut, Denmark). Tuberculin Purified Protein Derivative (PPD) (Statens Serum Institut, Denmark) is used to measure the immune response to persons either exposed to tuberculosis (TB) or vaccinated with BCG. The use of PPD combined with BCG has been postulated as an effective method for production of anti-hapten antibodies (De Silva, Egodage & Wilson, 1999).

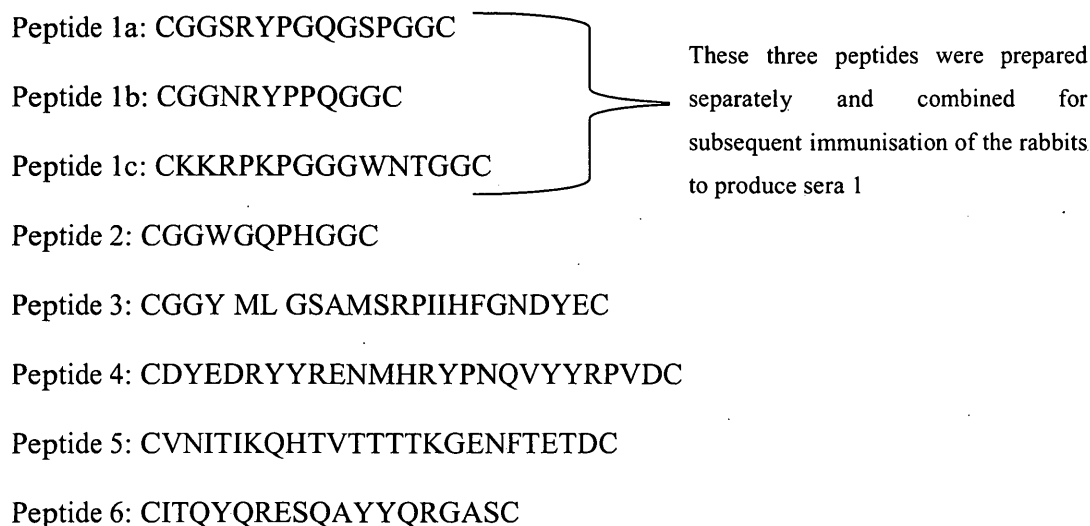


Figure 5-1 Synthetic peptide sequences for polyclonal antisera production

5.2.2 Polyclonal antibody production protocol

All peptides were commercially prepared (Severn Biotech) as above and also without the C-terminal cysteine residue (designated 1 cys and 2 cys) and both forms conjugated to PPD and keyhole limpet haemocyanin (KLH) (Sigma). The immunisation protocol was carried out by the Biological Investigations Group (BIG) at HPA-Porton Down. This protocol consisted of pre-immunisation of Dutch rabbits (approximately 2.5 kg) with 0.1 mL of reconstituted freeze dried BCG in two sites, following collection of sample of 1 mL of pre-immune sera. After a period of 4-6 weeks, the rabbits were inoculated with each peptide-PPD conjugate composed of 0.1 mg of each of peptide 1 (a,b,c in both 1 cys and 2 cys forms) or 0.3 mg each of the 1 cys and 2 cys forms for all the other peptides, dissolved in 1 mL 0.9 % sterile saline in an equal volume of Freund's incomplete adjuvant (Sigma). This was administered as 2 aliquots of 0.75 mL and 2 aliquots of 0.25 mL. The immune response was boosted by three further cycles of peptide-PPD conjugate injection of four 0.25 mL aliquots prepared as described and administered at 4-6 week intervals.

Three samples (~ 0.5-1 mL) of immune sera were taken 7-14 days after each boost cycle. The rabbits were immunised with 0.6 mg total of each peptide-PPD conjugate. After the final boost of antibody response which was confirmed by serum analysis by ELISA, terminal exsanguination was carried out and sera collected.

5.2.3 *Monitoring of antibody titre*

Antibody titres were monitored throughout by ELISA. Microtitre plates were coated with the appropriate synthetic peptide conjugated to an alternative carrier protein, bovine serum albumin (BSA) to exclude antisera produced to the carrier protein alone. KLH conjugated to appropriate peptides was coated at a concentration of 0.1 mg mL^{-1} in coating buffer (0.05M Sodium Carbonate buffer (BDH, UK), pH 9.6), (100 μL per well), overnight at room temperature, followed by a washing step and subsequent blocking with 3 % casein in PBS for 1 hour (200 μL per well). Anti-rabbit IgG peroxidase conjugate (Sigma) was added diluted in PBS+0.05% Tween-20 (PBS-T) and incubated for 1 hour at 37 °C. After washing, 100 μL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma) was added to the wells and incubated at room temperature for 15 minutes. The reaction was stopped by the addition of 100 μL of 1M sulphuric acid and absorbance (450 nm) read on a colourimetric plate reader (Multiskan Ascent, Thermo Life Sciences).

5.2.4 *Purification of polyclonal antisera*

The six antisera (denoted PAb 1-6) produced following terminal exsanguination were initially purified by ammonium sulphate precipitation, which was achieved by the addition of a volume of saturated ammonium sulphate solution (BDH, UK) to a concentration of 50% at 4 °C , followed by centrifugation at 13,500 rpm. The pellet was retained and re-

dissolved in PBS and then dialysed to remove ammonium sulphate. Following this step, each corresponding peptide was immobilised individually on a cross-linked agarose support matrix using a SulfoLink Kit (Pierce), according to manufacturer's instructions. The amount of peptide coupled was estimated and the efficiency of the reaction determined, enabling calculation of the molar equivalent of antibody to peptide. The peptide-affinity columns were then used for affinity purification of the polyclonal antibodies to their constituent peptides.

5.2.5 Characterisation of polyclonal antisera by ELISA

All the polyclonal sera (PAb1-6) were characterised by ELISA to determine specificity to purified recombinant PrP (rec PrP) (AbCam recombinant murine PrP). This was achieved by coating rec PrP onto a microtitre plate at a concentration of $0.1 \mu\text{g. mL}^{-1}$ and the plates subsequently blocked with 3% casein (skimmed milk powder), in PBS. Sera PAb1-6 and affinity purified sera PAb1-6 were diluted to an initial protein concentration of $25 \mu\text{g.mL}^{-1}$ and then diluted (doubling dilutions) to compare detection of rec PrP. A 1:20,000 dilution of anti-rabbit IgG peroxidase conjugate in (PBS-T) was added to the wells and incubated for 1 hour at 37°C . After washing, $100 \mu\text{L}$ of TMB substrate (Sigma) was added to the wells and incubated at room temperature (RT) for 15 minutes. The reaction was stopped by the addition of $100 \mu\text{L}$ of 1M sulphuric acid (Sigma) to each well and absorbance (450 nm) read on a colourimetric plate reader (Multiskan Ascent, Thermo Life Sciences).

5.2.6 Characterisation of polyclonal antisera by Western blot

A single infectious stock of mouse passaged BSE (301V) infectious mouse brain homogenate (iMBH) had been previously established by Dr Neil Raven and Mr Clive

Buswell, and kept stored at -80 °C. The method has been previously described (McLeod *et al.*, 2004, Taylor *et al.*, 2002). The stock was prepared by homogenisation of 48 infected mouse brains in 4 volumes of PBS and this sample was passed sequentially through increasingly fine gauge needles (Becton Dickinson) until free flowing. Normal mouse brain homogenate was prepared by the same method (MBH). Analysis of the detection specificities of PAB1-6 compared to 6H4 (Prionics) was achieved by SDS-PAGE followed by Western blotting. This was carried out using 4-12 % Bis-Tris NuPage gels in Mops electrophoresis buffer under reducing conditions (Invitrogen). Protein bands were visualised using Colloidal Blue Staining Kit (Invitrogen) and compared to Multi Mark molecular weight markers (Invitrogen). Samples of iMBH and protease digested iMBH were prepared by the addition of 10 % (v/v) of 20 mg mL⁻¹ protease solution, Purafect Ox (Genenecor) and incubation at pH 12 for 30 minutes with rec PrP included as a control. Western blotting was used to confirm the presence of immunoreactive bands. Protein bands resulting from SDS-PAGE electrophoresis were transferred to nitrocellulose using 400 mL of pre-prepared NuPage containing methanol (80 mL). Nitrocellulose membrane was pre soaked for 30 seconds in methanol before rinsing briefly in water and then soaked in ~50 ml transfer buffer for 5-10 minutes. Blotting pads and two pieces of filter paper in transfer buffer (no air bubbles) were pre-soaked prior to opening the gel cassette and removing the wells with a knife. The pre soaked filter paper was placed on the gel without trapped air bubbles. The surface of the gel was moistened with transfer buffer before positioning the presoaked nitrocellulose membrane on the gel. The sandwich was assembled in the transfer apparatus as shown in the NOVEX instructions. Fill the chamber with ~200 ml transfer buffer until the gel/membrane is just covered and the unit run at 20 V constant voltage for 40 min. Once transfer had been completed, the nitrocellulose membrane was removed and then blocked overnight in PBS-T + 3% casein. The membrane was incubated with either purified PAB1-6 described in section 5.2.4 (diluted 1:500) or the monoclonal antibody, 6H4 (Prionics) diluted 1:10,000 in PBS-T. After washing three times

with PBS-T, either a 1:1000 dilution of anti-rabbit IgG peroxidase (Sigma) or anti-mouse IgG peroxidase conjugate (Sigma) was added as appropriate and the membrane incubated for 1 hour at room temperature. Washing was repeated and the membrane visualised by the addition of TMB (Sigma).

5.3 Results

5.3.1 Characterisation of polyclonal antisera by ELISA

Antibodies were characterised by ELISA for their interaction with rec PrP (Figure 5-2, Figure 5-3 and Figure 5-4). Prior to affinity purification, three of the antibodies were shown to detect a concentration of $0.2 \mu\text{g. mL}^{-1}$ of rec PrP by ELISA. After affinity purification, all six polyclonal antibodies showed increased titres to rec PrP. In addition, PAb2, 3 and 5 showed a response to recombinant PrP after affinity purification, although they had not previously given a result above that of the negative control (Table 5-1).

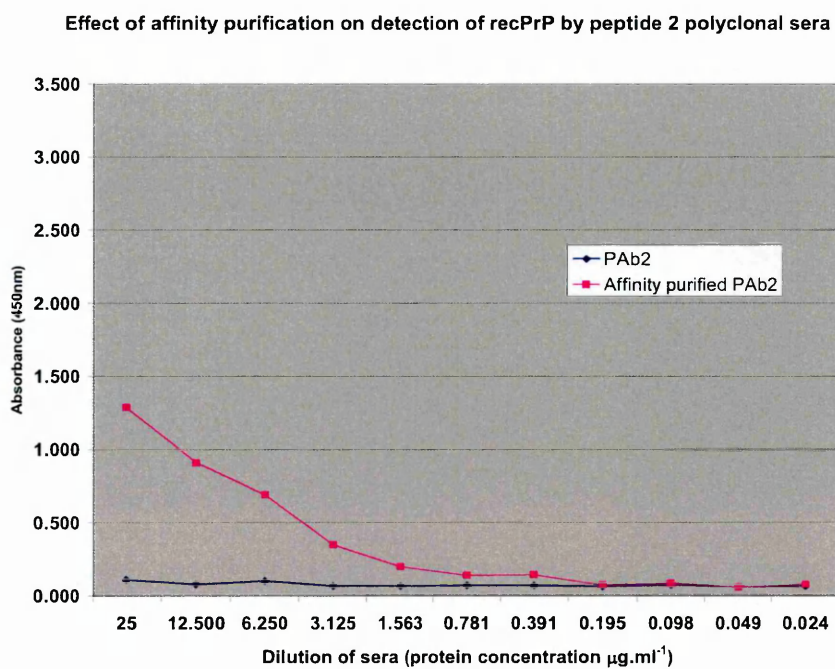
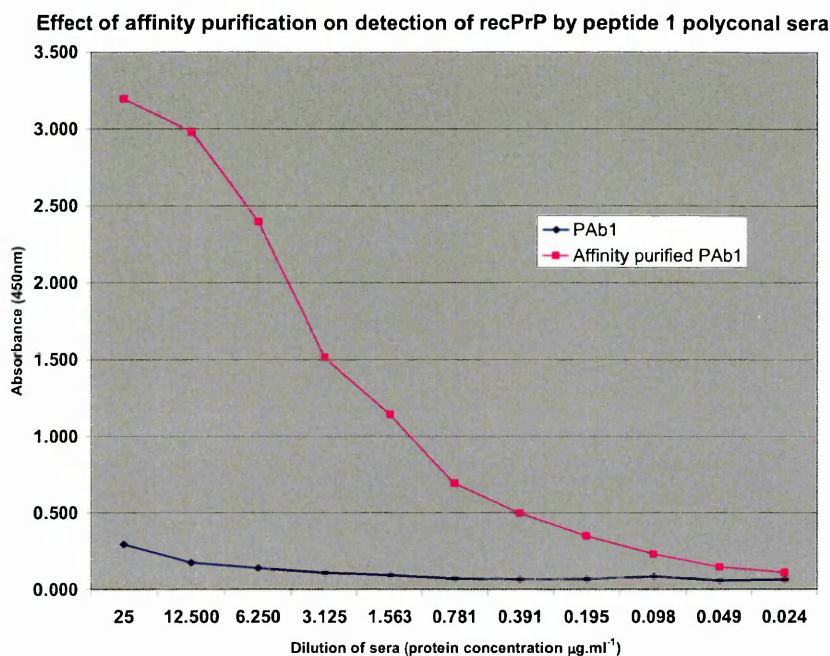


Figure 5-2 Detection of rec PrP by affinity purified PAb1 & 2

Rec PrP was coated at a concentration of $0.1 \mu\text{g. mL}^{-1}$ Sera PAb1 & 2 diluted to $25 \mu\text{g. mL}^{-1}$ and then diluted to compare detection of rec PrP. Anti-rabbit IgG peroxidase conjugate (Sigma) was added after washing steps and bound antibody conjugate detected with TMB substrate.

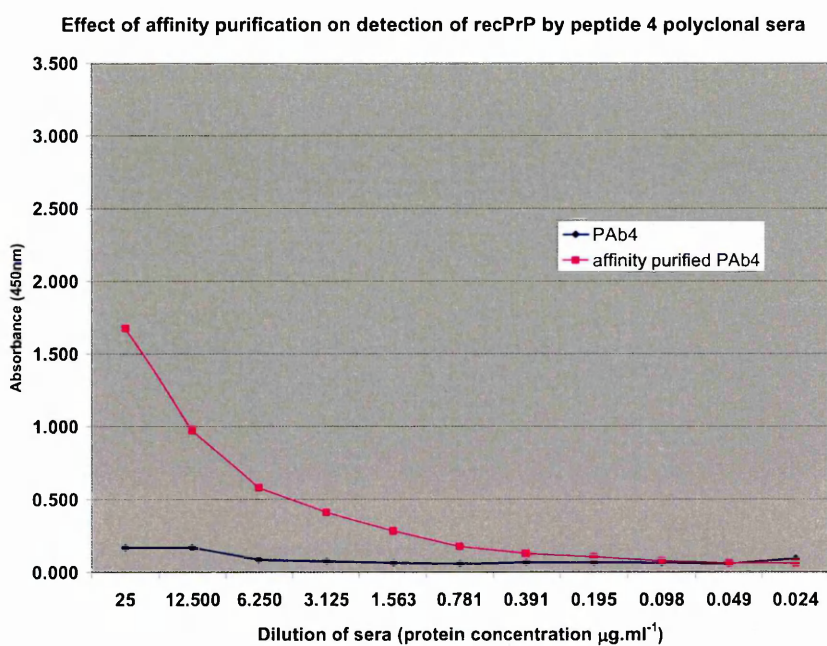
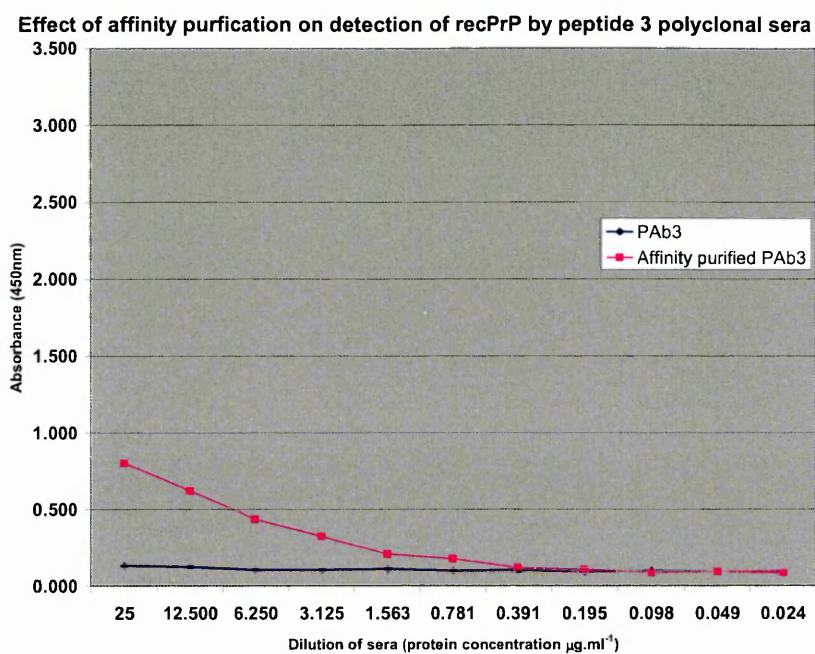


Figure 5-3 Detection of rec PrP by affinity purified PAb 3 & 4

Rec PrP was coated at a concentration of $0.1 \mu\text{g. mL}^{-1}$ Sera PAb3 & 4 diluted to $25 \mu\text{g. mL}^{-1}$ and then diluted to compare detection of rec PrP. Anti-rabbit IgG peroxidase conjugate (Sigma) was added after washing steps and bound antibody conjugate detected with TMB substrate.

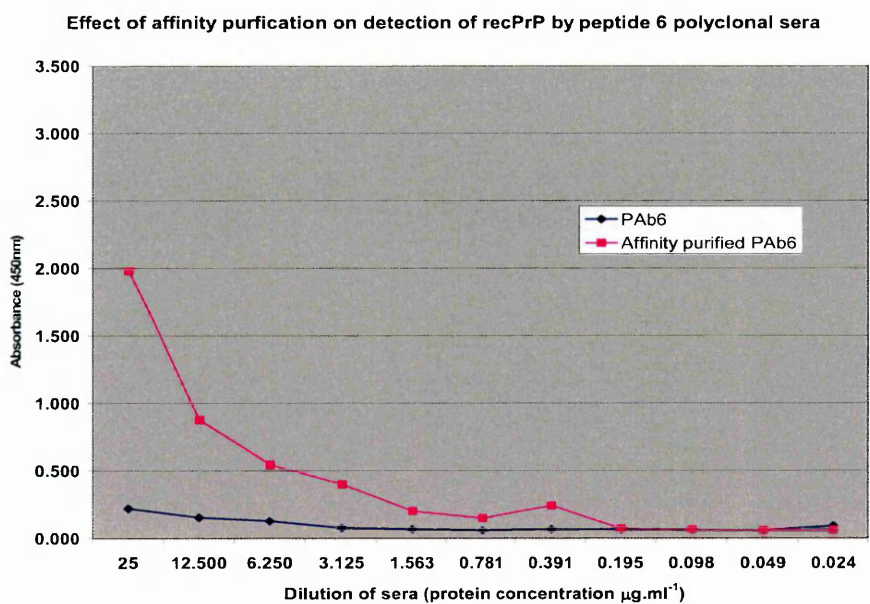
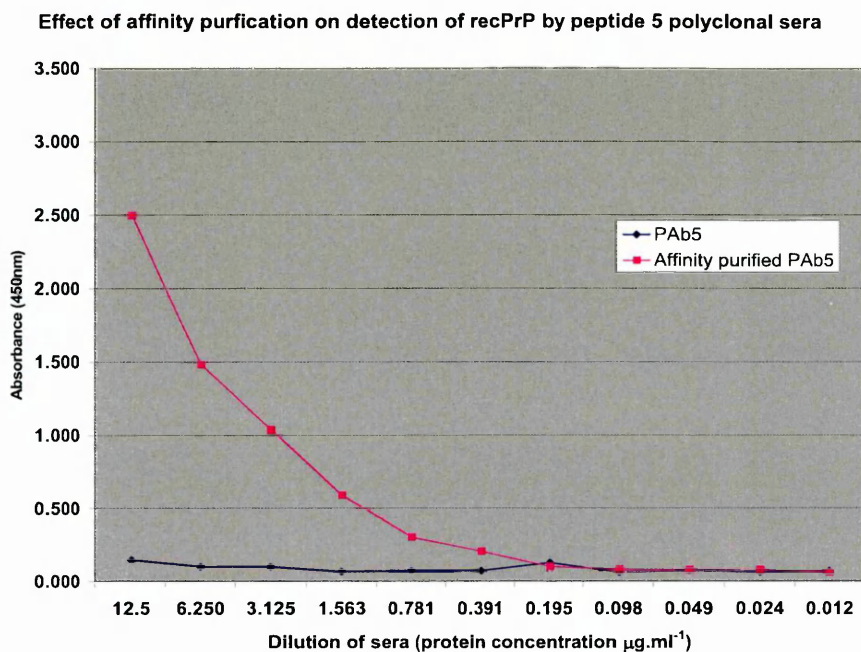


Figure 5-4 Detection of rec PrP by affinity purified PAb5 & 6

Rec PrP was coated at a concentration of $0.1 \mu\text{g. mL}^{-1}$ Sera PAb5 & 6 diluted to $25 \mu\text{g. mL}^{-1}$ and then diluted to compare detection of rec PrP. Anti-rabbit IgG peroxidase conjugate (Sigma) was added after washing steps and bound antibody conjugate detected with TMB substrate.

Polyclonal Sera Number	Sera ($\mu\text{g. mL}^{-1}$)	Affinity purified ($\mu\text{g. mL}^{-1}$)
PAb1	25	0.1
PAb2	-	3.1
PAb3	-	3.1
PAb4	12.5	0.8
PAb5	-	0.8
PAb6	25	0.2

Table 5-1 Comparison of detection of rec PrP by PAb.

Note: the lower figure denoting increase in sensitivity of detection.

5.3.2 Characterisation of polyclonal antisera by Western blot

It was clear that the anti-prion antibodies derived from the in-house polyclonal antisera, PAb1-6, demonstrated specificity in respect to rec PrP in both ELISA, although an affinity purification step was required in order for a number of the antisera. Only one antibody (PAb3) recognised the putative PrP^{Sc} monomer bands in corresponding Western blots employing BSE (301V) infectious mouse brain homogenate (Figure 5-5). Four of the antibodies (PAb2, PAb3, PAb5 and PAb6) identified protease-digestion resistant bands of higher molecular weight (up to 9 bands having an apparent molecular weight range of approximately 40-70 kDa). PAb1 and PAb4 by contrast failed to identify any bands in protease-digested BSE infectious samples.

These results, which are summarised in Table 5-2 were initially promising as detection of rec PrP had been achieved to some extent by all of the antibodies by ELISA and Western blot and levels of detection on ELISA had been substantially improved by affinity purification of the antisera. However, the results of the Western blots were disappointing because although four of the antibodies (PAb2, PAb3, PAb5 and PAb6) identified protease resistant bands of higher molecular weight, (up to 9 bands having an apparent molecular weight range of approximately 40-70kDa) only PAb3 identified the highest of the characteristic PrP^{Sc} bands within the infectious MBH sample.

Polyclonal sera	rec PrP	iMBH
PAb1	√	
PAb2		
PAb3		√
PAb4	√	
PAb5		
PAb6	√	
6H4	√	√

Table 5-2 Summary of detection of recPrP and iMBH by polyclonal antisera and 6H4

5.4 Discussion

The availability of anti-prion antibodies that are capable of preferentially recognising the protease resistant form of PrP (PrP^{Sc}) remains a significant issue for the development of immunoassays (Gilch *et al.*, 2003). The cost of commercially available antibodies is generally prohibitive and therefore for the progress of this project it was considered important to attempt to generate a source of in-house antibodies for use in the development of the assay. It is generally recognised that PrP is poorly immunogenic and it has been hypothesised to be due to the ubiquitous presence of cellular prion proteins and the failure of the immune system to generate auto-antibodies (Prusiner *et al.*, 1993). Therefore the aim was to employ a strategy designed to boost antibody responses to the prion mimetic peptides, to attempt to overcome the problems associated with generation of anti-prion antibodies.

An approach which has been previously been taken was the use of cyclic presentation of peptides to potentially enhance the immune response by providing some conformational similarity to native protein structure (De Silva, Egodage & Wilson, 1999; van der Werf *et al.*, 1994). The peptides were therefore synthesised with N and / or C-terminal cysteine residue in order that both cyclic and linear peptides would be presented on conjugation to a carrier protein.

In addition, an alternative method was employed that utilised an alternative carrier protein, protein purified protein derivative (PPD), in conjunction with pre-immunisation with BCG. This had previously been described by researchers as a technique to improve the serum antigen response and production of hapten specific polyclonal and monoclonal antibodies. PPD is used as a measure of T-cell response to either tuberculosis or BCG and had

therefore been suggested for use as an alternative carrier protein (De Silva, Egodage & Wilson, 1999). The use of this approach was therefore adopted within this study.

Characterisation of the anti-PrP polyclonal sera produced using these strategies showed moderate to high titres against the immunogen itself. Further analysis by ELISA demonstrated that after affinity purification that all the polyclonal sera (PAb1-6) detected rec PrP to some extent and therefore it was anticipated that these antisera would be useful in the development of ultra-sensitive assay for detection of rec PrP and infectious prion material. It was clear, however on further characterisation by Western blot that whilst PAb1, 3, 4, 5 and 6 were able to recognise rec PrP, only PAb 3 was able to recognise the putative PrP^{Sc} monomer in Western blots employing BSE (301V) infectious mouse brain homogenate (iMBH).

PAb3 and three further antibodies (PAb2, PAb5 and PAb6) also recognised a series of up to nine higher molecular weight bands (approximately 40-60 kDa) in protease-digested MBH, and with no comparable high molecular weight bands observed in non-infectious MBH, whereas the antibodies PAb1 and PAb4 failed to identify any bands in protease-digested BSE infectious samples. It was hypothesised that these higher molecular weight bands were potentially significant and additional research was carried out which did not form part of this study, however it was eventually identified that these bands were probably caused by the presence of a bacterial contaminant in the original infectious MBH sample.

In both ELISA and Western blots, the commercial antibody 6H4 showed significantly better detection of rec PrP than the in-house polyclonal antisera and additionally showed detection of the characteristic PrP^{Sc} bands within the protease digested infectious MBH. It was therefore apparent that despite the use of strategies to attempt to increase the success

of the production of specific anti-prion polyclonal antibodies that the antisera produced were of limited use for further development of the assay.

It was therefore decided that further development of the ultra-sensitive prion ELISA would focus on the use of the commercially available monoclonal antibody 6H4, with the advantage that it would enable comparison of the detection limits achieved with other published material (Barletta *et al.*, 2005; Biffiger *et al.*, 2002; Lourenco *et al.*, 2006).

6 Development of ultra-sensitive prion AK-ELISA

6.1 Introduction

The potential for iatrogenic transmission of CJD through a number of routes such as surgery and blood transfusion continues to be identified as an issue (reviewed in Grassi *et al.*, 2008 and Ironside, 2006). Consequently there remains an urgent requirement for development of reliable and suitable processes for inactivation of prions for inclusion within sterile service departments (SSDs) within the NHS. Alongside development of novel processes for removing prion infectivity on surgical instruments, there is a requirement for methods of validating this process. In addition there is evidence of transmission of vCJD through blood transfusion which has highlighted the requirement for a sensitive detection assay suitable for screening of blood (Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006).

Attempts by researchers to develop assays with suitable levels of sensitivity required have been hampered by lack of specific antibodies for the disease associated form of the prion protein. It was therefore decided that the priority was to focus on development of the detection end of the assay to enhance the overall sensitivity as far as possible. Therefore the potential use of a novel AK reporter enzyme was explored the possibility of increased detection of prion proteins within large excesses of biological material such as neuronal tissue. There are a number of decontamination methodologies currently being developed (Baxter *et al.*, 2005; Fichet *et al.*, 2007; Heindl *et al.*, (in press); Jackson *et al.*, 2005; McLeod *et al.*, 2004; Whittaker *et al.*, 2004) and therefore there is clearly a requirement for a method of validation of these processes which would be suitable for use in settings such as SSDs.

The focus of this phase of the study was to use the previous findings to enable further development of a flexible assay format to deal with the specific requirements of TSE detection. One of the main aims was optimisation of all aspects of the novel assay format to enhance sensitivity of detection as far as possible.

In order to optimise the prion AK-ELISA all assay conditions were identified for full evaluation and these included:

1. Identification of optimal dilutions of antibody-AK conjugate and incubation conditions.
2. Optimisation of reduction of non-specific binding of antibody-AK conjugates by:
 - a. Comparison of blocking methods including casein, bovine serum albumin, (BSA) and various commercial blocking reagents (Sea Block, SuperBlock (Pierce)).
 - b. Analysis of presence of detergent e.g. Tween-20, in the conjugate diluent
3. Identification of optimal conditions for thiol- cleavage of the AK moiety from bound conjugate including assessment of:
 - a. Various cleavage methods using increasing concentrations of Dithiothreitol (DTT), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP.HCl) and 2-Mercaptoethanesulfonic acid (MESNA). Efficacy of transfer was monitored by assessing residual AK activity in comparison to the transferred AK activity.

- b. Use of different buffers and pH conditions e.g. carbonate, borate, phosphate and tris buffers in addition to PBS. The pH conditions assessed were relevant to the buffering capacity of the various solutions assessed.
 - c. The efficacy of cleavage was assessed at varying temperatures (20-70 °C).
- 4. Assessment of conditions to optimise maximise ATP generation by AK;
 - a. Optimisation of the concentration of the ADP substrate required by assessing a full range of concentrations (1µM – 1mM).
 - b. Assessment of effect of temperature (37-80 °C) on generation of ATP
- 5. Optimisation of reduction of the non-specific background signal produced by contaminating AK and ATP present in tissue.
 - a. Determination of the optimal temperature and duration of heat inactivation step to effectively reduce background signal without affecting the activity of the thermostable AK. A range of temperatures from 50-90 °C were examined over a time course of 5- 30 minutes.
 - b. Assessment of the conditions required for effective ATP hydrolysis by ATPase (apyrase) followed by method of inactivation of apyrase prior to completing the assay. A range of conditions such as the concentration of apyrase required; the length of incubation followed by assessment of a range of temperatures to inactivate the apyrase (37-70 °C) were examined.

Further optimisation was carried out prior to performing the assay described (6.3.7) including:

6. Optimisation of practical aspects of assay:
 - a. Comparison of methods used for heat step including evaluation of heat block versus PCR thermocycler.
 - b. Evaluation of the use of plate luminometer injectors for delivery of luciferin-luciferase reagent and impact on results.
7. Establishment of internal controls for use in all AK-ELISA assays

Evaluation of assays performed in order to establish a method of calculating a reliable assay cut-off point for identification of detection limits. The optimised AK-ELISA would then be used to assess limits of detection of rec PrP and infectious prion material on the surface of surgical steel and within the presence of biologically relevant material and compared to the use of 6H4 in other published studies and also with a traditional HRP assay format.

6.2 Materials and Methods

6.2.1 Conjugation of 6H4 to thermostable adenylate kinase

As discussed in Chapter 5, it was decided to concentrate on the use of a commercially available antibody, 6H4 (Prionics) for the purposes of optimisation and evaluation of the novel assay format for detection of prion protein.

A 10 mg stock of freeze dried thermostable *Sulfolobus acidocaldarius* adenylate kinase was reconstituted in 1 mL of distilled H₂O and then dialysed against PBS (2 x 4 L) overnight at 4 °C. 0.3 mg of the water soluble and long chain form of the

heterobifunctional reagent, N-Succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-SPDP) (Pierce) was added directly to the sample and stirred for 1 hour at room temperature. The derivatised AK in solution was transferred to pre-prepared dialysis tubing and dialysed extensively against PBS overnight at 4 °C to remove unreacted SPDP. 6H4 was supplied as 1 mg of purified antibody within 0.5 mL of buffer and subsequently derivatised by the direct addition of 0.3 mg of the sulfo-LC-SPDP to this solution and stirring for 1 hour at room temperature. Unreacted SPDP was removed by dialysis against PBS. Concentration of protein was estimated using by Biorad Protein Assay and absorbance (595nm). The derivatised AK was reduced by the addition of dithiothreitol (DTT) followed by incubation for 30 min at room temperature. The DTT was removed by passing the sample down a PD10 column (Pierce) pre-equilibrated with PBS and 1 mL fractions collected. A 5:1 ratio of AK to IgG was calculated using molar ratios and the two derivatised solutions combined. The reaction was stirred overnight at room temperature. The conjugation reaction was stopped by the addition of 10 μ L of 5 mg.mL⁻¹ solution of N-ethyl maleimide (Sigma) freshly prepared in anhydrous DMSO followed by incubation for 30 minutes at room temperature and the unreacted N-ethyl maleimide removed by dialysis against PBS. The conjugate preparation was stored frozen at -20 °C.

6.2.2 Detection of rec PrP by optimised prion ELISA

Rec PrP (murine PrP, AbCam) was diluted to a starting concentration of 500 ng.mL⁻¹ and diluted in carbonate coating buffer, pH 9.6 across the wells of a microtitre plate (Nunc©MaxiSorp) 100 μ L / well (in duplicate) and incubated overnight at room temperature. The assay was controlled by inclusion of wells omitting rec PrP, 6H4-AK and ADP substrate respectively. The plate was sealed using a clear plate sealer (Greiner) for all incubation steps to protect from contamination or loss of volume.

All washing steps were carried out on a Wellwash AC 96 well plate washer and wells washed (x4) with 300 μ L PBS-Tween 0.05% (PBS-T). After initial coating of the antigen, unbound antigen was removed prior to blocking of non-specific binding by the addition of 300 μ L per well of 5% casein diluted in PBS-T and incubated for 30 minutes at 37 °C. Following an additional wash step a 1:2,000 dilution of 6H4-AK conjugate diluted in 5% casein / PBS-T was added followed by incubation for 1 hour at room temperature while shaking gently at ~150 rpm in a microtitre plate incubator / shaker (Heidolph Titramax 1000). Following incubation with conjugate, the plate was washed prior to addition of a 25 mM solution of 2-mercaptoethane sulfonate (MESNA) (Sigma) diluted in 50 mM Tris-HCl, pH 7.2 which was further incubated at 45 °C for 30 minutes. The contents of the plate were transferred to a white luminometer plate using a multi-channel pipette. The plate was placed in a thermocycler or heat block (Eppendorf) for the heat inactivation step at 80 °C for 20 minutes to inactivate contaminating thermolabile AK, followed by addition of 5 μ L / well of apyrase (Luminase, Celsis, reconstituted in PBS) for inactivation of contaminating ATP present prior to ATP generation. 100 μ L of 135 μ M ADP (Celsis) prepared in 15 mM MgAc, 1 mM EDTA, pH 6.8 and added to appropriate wells followed by incubation for 20 min at 70 °C using a PCR thermocycler or heat block (Eppendorf). ATP reagent (Biothema L / L reagent) was added to each well (30 μ L) either using a multi-channel pipette or Luminometer injection system (Berthold Orion) before reading of the relative light units (RLUs) generated by ATP.

6.2.3 Detection of PrP in situ on the surface of surgical instruments

Surgical grade stainless steel disks of (5 mm diameter) (Goodfellow; Cambridge Ltd) were placed and glued in the bottom of a microtitre well. These were coated by carefully placing

solution of rec PrP and / or non-infectious mouse brain homogenate (MBH) in coating buffer (100 μ L) on the surface of the disk and incubating overnight at room temperature. The unbound antigen was removed by washing with PBS-T before addition of 100 μ L of a 1:2,000 dilution of 6H4-AK conjugate within PBS-T + 5% casein and incubated for 1 hour at room temperature. After another wash step, the AK was cleaved from the bound conjugate by treatment with 100 μ l of 25 mM MESNA in 50 mM Tris -HCl pH 7.2 at 45 °C for 30 minutes. The contents of the microtitre well were transferred to a white thermocycler compatible microtitre plate. Thermal inactivation of contaminating AK was achieved by heating to 80 °C for 10 minutes. Apyrase (Celsis) (5 μ L) of was added to each well and incubated at 37 °C for 30 minutes followed by inactivation of the apyrase by incubation at 65 °C for 10 minutes. 100 μ L of 135 μ M ADP (Celsis) prepared in 15 mM MgAc, 1 mM EDTA, pH 6.8.was added to appropriate wells and incubated for 20 min at 70 °C using a heat block (Eppendorf). The luminometer injection system was primed with ATP reagent (Biothema L / L reagent). The microtitre plate was cooled to room temperature before addition of 30 μ l per well.

6.2.4 Detection of rec PrP present in neuronal tissue

This assay was carried out as the method described above (6.2.2), with the exception that rec PrP was diluted into a two different concentrations of non-infectious mouse brain homogenate (MBH) at 5,000 and 50,000 fold excess. The assay was then completed as described (6.2.2).

6.2.5 *Detection of rec PrP present in whole blood*

This assay was carried out as the method described above (6.2.2), with the exception that rec PrP was diluted into sheep's blood (TCS Biosciences). The assay was then completed as described (6.2.2).

6.2.6 *Detection of rec PrP present in sera*

This assay was carried out as the method described (6.2.2); with the exception that rec PrP was diluted into undiluted sheep's sera (Sigma). The assay was then completed as described (6.2.2).

6.2.7 *Detection of infectious MBH (non-proteinase K digested)*

A single infectious stock of mouse-passaged BSE (301V) infectious mouse brain homogenate was established as described (McLeod *et al.*, 2004; Taylor *et al.*, 2002) by Dr Neil Raven and Mr Clive Buswell. Titration of this material by dilution and re-inoculation into indicator mice identified a titre of approximately $10^{8.9}$ infectious units per gram mouse brain. A batch non-infectious mouse brain homogenate was prepared and stored. The iMBH was diluted from a starting concentration of 1 mg.mL^{-1} in coating buffer (100 μL) and incubated overnight at room temperature. The assay was then completed as described in section 6.2.2.

6.2.8 Detection of iMBH in situ on surface of surgical steel disks

The infectious mouse brain homogenate (iMBH) was prepared as described previously (6.2.7). Surgical grade stainless steel disks of (5 mm diameter) (Goodfellow; Cambridge Ltd) were placed in the bottom of a microtitre well. Infectious mouse brain homogenate was prepared as described (6.2.7). The iMBH was diluted from a starting concentration of 1 ng.mL^{-1} and diluted in coating buffer (100 μL) and incubated overnight at room temperature. The assay was then completed as described in section 6.2.2.

6.2.9 Comparison of assay formats

Detection of rec PrP using 6H4 within an HRP assay format was carried out by the following method. Rec PrP (recombinant murine PrP, AbCam) was diluted to a starting concentration of 500 ng.mL^{-1} and diluted in carbonate coating buffer, pH 9.6 across the wells of a microtitre plate (Nunc©MaxiSorp) (100 μL) followed by overnight incubation at room temperature. The assay was controlled by the addition of three controls which omitted antigen; primary antibody; and secondary antibody respectively. The plate was sealed with a clear plate sealer (Greiner) to protect from contamination or loss of volume during incubation and heat steps.

All washing steps were carried out on a Wellwash AC 96 well plate washer and wells washed (x4) PBS-T. The plate was washed prior to blocking of non-specific binding by the addition of (300 μL) of 5% casein diluted in PBS-T to the wells and incubation for 30 minutes at 37 °C. A 1:5000 dilution of 6H4 (Prionics) was added to the wells (100 μL) diluted in PBS-T + 5% casein and incubated at 37 °C for 1 hour. The solution was removed by washing wells, before addition of 1:20, 000 dilution of anti-mouse IgG

peroxidase conjugate (Sigma) which was incubated for 1 hour at 37 °C. Following incubation the wells were washed as previously described and then tetramethylbenzidine (TMB) substrate (Sigma) was added (100 µL) and incubated for 15 minutes at room temperature. Colour development was stopped by the addition of 1M sulphuric acid (100 µL). Absorbances (450nm) were read using Multiskan plate reader (Labsystems).

6.3 Results

6.3.1 *Optimisation of AK- ELISA detection of rec PrP*

As discussed in section 6.2.2 it was decided that further development of the assay would focus on the use of rec PrP in combination with 6H4-AK conjugate as previous results obtained had shown that this conjugate achieved the most sensitive detection of rec PrP. This gave an additional benefit in that it allowed the optimisation stages to be carried out without the requirement for the use of infectious material and containment facilities. The parameters evaluated to optimise the assay are fully described in section 6.2.2 and the results are as follows:

1. Optimal dilutions of conjugate and incubation conditions.

The optimal dilution of 6H4-AK conjugate was determined as a dilution of 1:2,000 dilution with PBS-T containing 5% casein followed by incubation for 1 hour at room temperature.

2. Optimisation of blocking of non-specific binding of the antibody-AK conjugates.

Optimal blocking of non-specific binding was obtained using a solution of 5% casein in PBS-T and incubation for 30 minutes at 37 °C.

3. Identification of optimal conditions for cleavage of AK from bound conjugate.

The results were assessed by examination of the residual AK activity versus transferred AK following transfer and washing of plate, which enabled comparison of the efficacy of the various methods assessed. All of the methods assessed resulted in approximately 80-85% cleavage of the AK. The use of MESNA was chosen ultimately because it is relatively inexpensive and non-toxic at a concentration of 25 mM and incubation at 45 °C for 30 minutes.

4. Optimal ATP generation by AK

The optimal quantity of ADP was determined to be as 100 µL of a 135 µM solution of ADP (Celsis) diluted in magnesium acetate buffer containing 1 mM EDTA with optimal incubation defined as 70 °C for 20 minutes.

5. Optimisation of reduction of non-specific background signal by contaminating AK and ATP present in tissue.

A heat treatment step of 80 °C for 10 minutes was sufficient to inactivate contaminating thermolabile AK activity even from vast excess of biological material, without affecting the activity of the recombinant *Sulfolobus acidocaldarius* AK. 5 µL of apyrase per well followed by, incubation at 37 °C for 30 minutes was sufficient to inactivate even high quantities of ATP present in the solution and use of heat treatment at 60 °C for 10 minutes to subsequently inactivate the apyrase prior to ATP generation.

Controls were established to be included in every assay by exclusion of antigen, 6H4-AK conjugate and ADP substrate sequentially. In addition, a control of ATP standard (Biothema) was also included to ensure that the luciferin luciferase reagent was within an

optimal range of RLUs and to allow reliable comparison between assays performed at different times.

6.3.2 Detection of rec PrP by fully optimised AK- ELISA

All the various conditions of the assay were fully optimised as described in sections 6.2.2 and 6.3.1 using detection of a constant concentration of rec PrP with 6H4-AK. This established format was then used to determine detection limits of the assay specifically for rec PrP alone, before comparison within different biologically relevant substrates such as neuronal tissue, blood and sera and on the surface of stainless steel.

The first objective was therefore to establish sensitivity for detection of rec PrP coated directly on a microtitre plate and the assay was completed as described (6.2.2). The results shown in Figure 6-1 demonstrate that detection of 15.6 pg.mL^{-1} of rec PrP was achieved, which equates to 0.67 pM (670 fM) of rec PrP. Statistical analysis was performed using the Minitab programme and a paired T-test gave a probability (P) value of 0.012 (deemed significant if less than 0.05) therefore demonstrating that this detection value in respect to the control was statistically significant. The use of statistics in repeated assays was used to establish a method of determining a cut-off value and this was deemed as the value of the control plus 3 times the standard deviation.

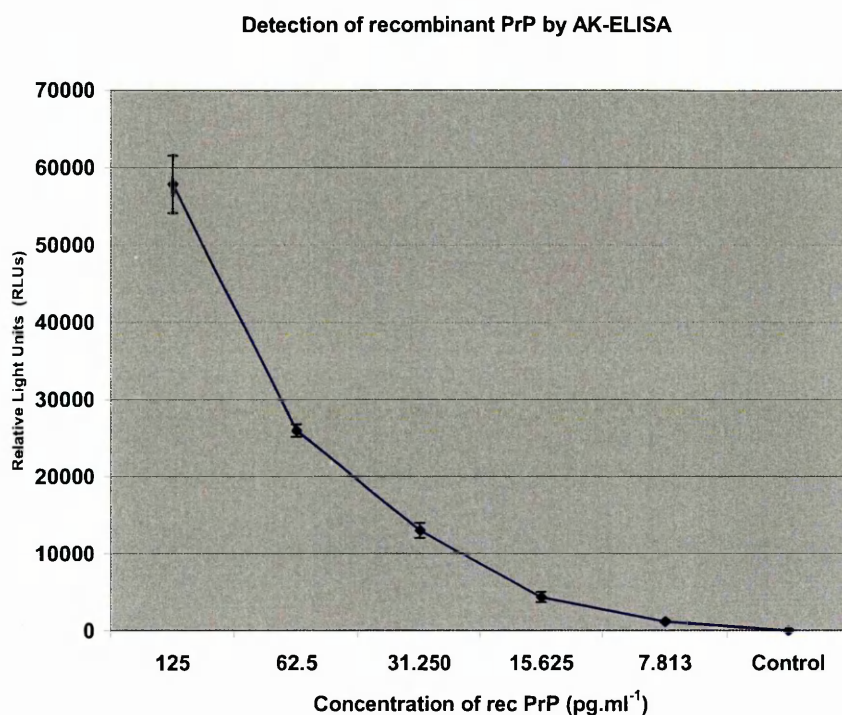


Figure 6-1 Detection of rec PrP by PrP AK- ELISA.

Rec PrP was coated onto well of a microtitre plate from a starting concentration of 125 pg.mL⁻¹ and diluted by doubling dilutions and the protein detected using 6H4-AK conjugate as described. The assay detected 15.6 pg.mL⁻¹ and the control shown to be statistically significant. (Paired T-Test giving a P value of 0.012). The figures were adjusted to the control. (control = 0).

Paired T-Test for 15.625 - control

	N	Mean	StDev	SE Mean
15.625	3	4388	831	480
control	3	0	0	0
Difference	3	4388	831	480

95% CI for mean difference: (2324, 6453)

T-Test of mean difference = 0 (vs not = 0): T-Value = 9.15 P-Value = 0.012

6.3.3 Detection of rec PrP on surfaces.

The next main objective was to determine if the use of this novel AK format would enable detection of rec PrP *in situ* on the surface of surgical grade stainless steel in order to explore the potential of the developed method for use for validation of cleaning and sterilisation processes specifically for prion infective material. This assay which utilised surgical grade stainless steel disks was therefore designed as a model for this use and the assay carried out as described (6.2.3). The rec PrP was allowed to fully dry prior to completing the assay in order to mimic potential conditions encountered following surgery. In Figure 6-2, the results show that $\sim 40 \text{ ng.mL}^{-1}$ rec PrP ($\sim 1.6 \text{ nM}$) can be detected on surfaces relevant to surgical instrument decontamination *in situ*.

However, compared to the results demonstrated for direct detection of rec PrP (Figure 6-1) the results were less sensitive with detection of nanogram levels compared to picograms. In addition the graph produced shows that the results on dose response were not linear. It was hypothesised that this may be due to the binding characteristics of rec PrP to stainless steel, which has not been optimised for binding of proteins unlike that of commercially available microtitre plates. It was hoped that use of infectious material may be an improvement due to the nature of the infectious agent which is known to adhere efficiently to surfaces such as stainless steel. It was decided therefore, to concentrate on the potential use of the assay format for detection within blood and tissue until the *in situ* detection could be repeated using BSE (301V) infectious MBH. This delay was due to the lack of availability of suitable containment facilities until a relatively late stage of this study.

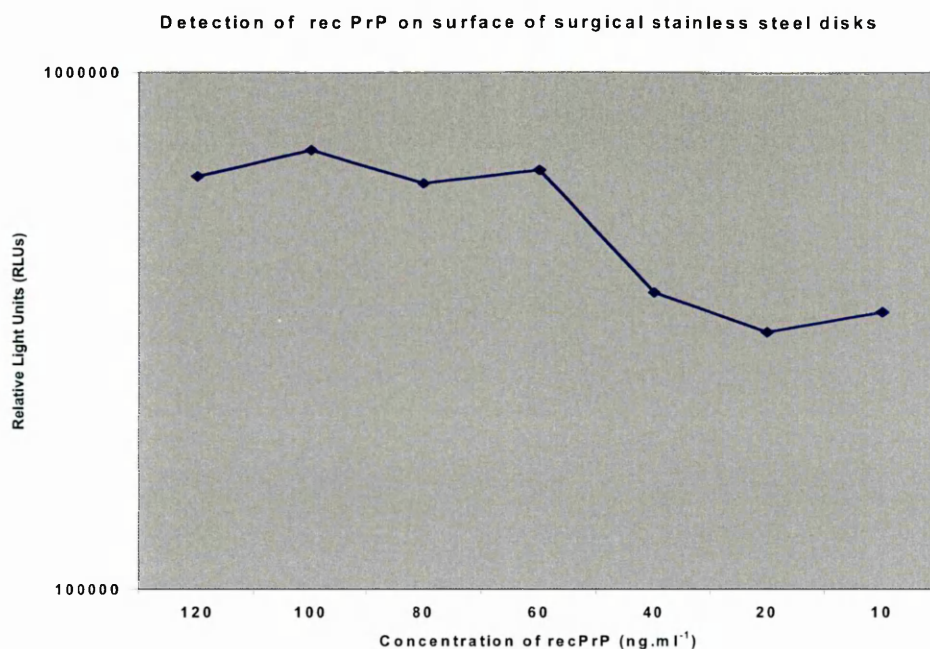


Figure 6-2 Detection of rec PrP on the surface of surgical stainless steel disks.

Rec PrP was coated onto surgical-steel grade disks and the presence of bound antigen detected by 6H4-AK conjugate followed by the standard AK-ELISA. The figures were adjusted to control. (control = 0).

6.3.4 Detection of rec PrP in a background of neuronal tissue

The previous results demonstrated that the developed assay format was capable of very sensitive detection of recombinant prion material using the AK-ELISA for direct detection within a microplate format, and to some degree of detection on the surface of surgical steel. It was therefore decided to evaluate the potential of this methodology for detection of recombinant prion material within complex biological samples. Mouse brain homogenate (MBH) was used to simulate these conditions, as this provided a good example of the type of tissue expected to be found on surgical instruments potentially contaminated with CJD. In order to fully evaluate the sensitivity of detection of an antigen within this type of substrate, two dilutions of non-infectious mouse brain homogenate (MBH) were used, to simulate worse case scenario of detection within a vast excess of biological tissue and this

was used to assess the limits of the assay. Figure 6-3 demonstrates the results of this assay with detection limits of rec PrP within both samples containing MBH at 5,000 and 50,000 fold excess calculated as 3.91 ng.mL^{-1} (cut-off value calculated to be 7360 RLU). This result demonstrated that utilisation of the thermostable properties of the AK reporter had effectively removed all contaminating AK prior to ATP generation and detection even from samples containing a vast excess of material. The detection of $\sim 400 \text{ pg}$ of rec PrP was less than previously seen, furthermore on additional analysis of the results showed that the RLU value for this concentration of rec PrP was $\sim 20,000$ therefore there is clearly the potential for more sensitive detection of rec PrP within this existing format.

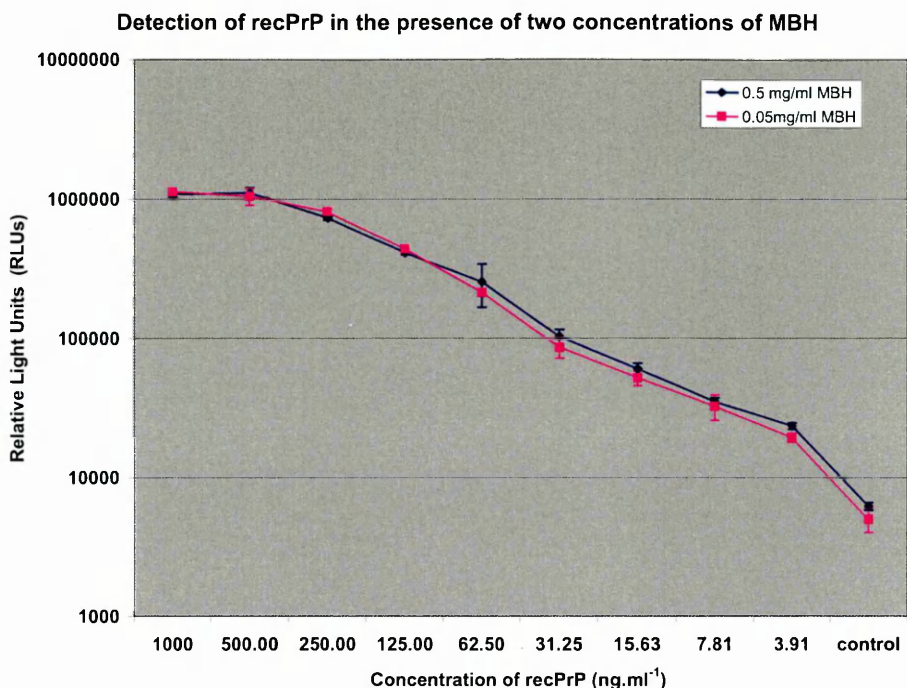


Figure 6-3 Detection of rec PrP in the presence of two concentrations of MBH.

Rec PrP was diluted in the presence of two concentrations of mouse brain homogenate and bound to an ELISA plate. The rec PrP was detected using 6H4-AK, followed by ATP generation and bioluminescent detection. The assay shows a limit of detection of 3.91 ng. mL⁻¹ with the assay unaffected by the presence of different excesses of MBH. The detection limit is calculated as negative control + 3 x standard deviation; the cut-off was established as 7360)

6.3.5 Detection of rec PrP within whole blood

It has been identified within recent publications that there remains an urgent requirement for the development of a blood test, both as a diagnostic and for screening for blood donations (reviewed in Grassi *et al.*, 2008 and Ironside, 2006). It was therefore decided that one focus of the research study would be to evaluate the potential of the developed AK-ELISA for this purpose. Sheep's blood was used as it is readily available

commercially and it was important to determine if the sensitivity of detection would be affected by the presence of whole blood components within the assay format.

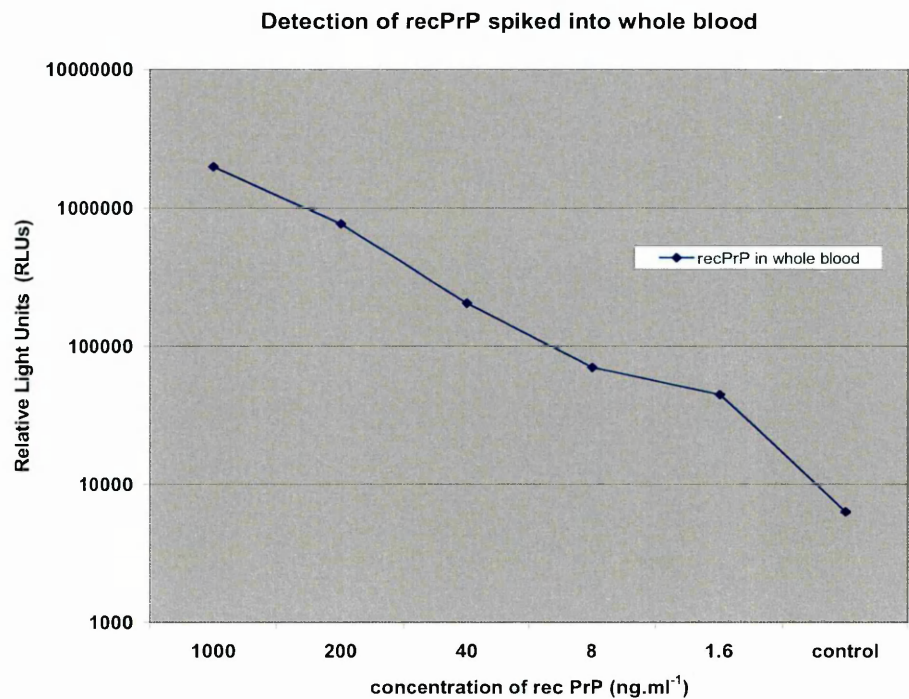


Figure 6-4 Detection of rec PrP spiked into whole blood.

Rec PrP (1000 ng) was diluted (5-fold dilutions) in the presence of sheep’s blood and bound to a microtitre plate The rec PrP was detected using 6H4-AK, followed by ATP generation and bioluminescent detection The assay shows a limit of detection of 1.6 ng.mL⁻¹ (The detection limit is calculated as negative control + 3 x standard deviation and cut-off calculated as 10495)

This assay was carried out by spiking a dilution of rec PrP within blood (6.2.5) and thereafter the assay was completed as standard and the results are shown in Figure 6-4. The results show sensitive detection of 1.6 ng.mL⁻¹ rec PrP using this assay format within a substrate of whole blood. The cut-off value was calculated as ~10,000 RLU and as the RLU value for the lowest concentration assessed was ~40,000 RLU this again showed

potential for more sensitive detection by further titration of rec PrP, even without further optimisation of the assay.

6.3.6 Detection of rec PrP within serum

In order to further explore the potential of the assay to detect prion material within blood and blood products, it was decided that blood sera would be used as a diluent for rec PrP to assess the sensitivity of the assay within this substrate. Sheep's serum was used for the assay due to the commercial availability. The assay was carried out as described in 6.2.6 and the results are shown in Figure 6-5. The limits of detection were assessed using the calculated cut-off value as 1.6 ng.mL^{-1} . The results of the assays described demonstrate that the use of the thermostable AK within the novel format is sufficient to effectively remove contaminating AK and ATP present in blood and sera without compromise of the assay sensitivity.

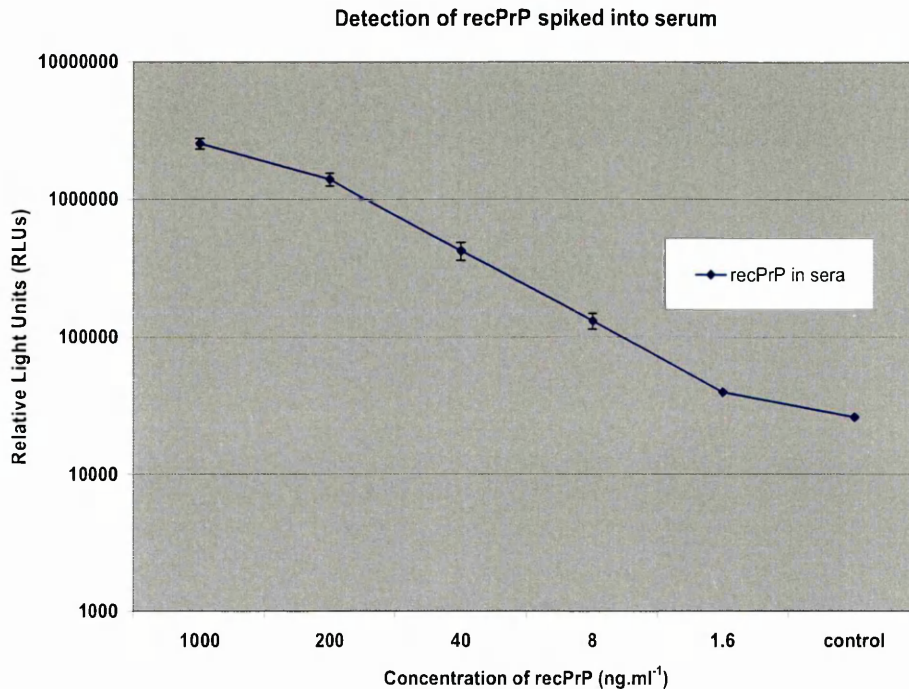


Figure 6-5 Detection of rec PrP spiked into serum.

Rec PrP was diluted in the presence of sheep's sera and detected using the AK-ELISA format. The rec PrP was detected using 6H4-AK, followed by ATP generation and bioluminescent direction. The assay shows a limit of detection of 1.6 ng.mL⁻¹ (The detection limit is calculated as negative control + 3 x standard deviation and calculated as 10495)

6.3.7 Detection of iMBH (non-proteinase K digested)

It had been shown that sensitive detection of recombinant prion protein was possible alone or within biological substrates such as neuronal tissue, blood and serum. It therefore remained to explore the potential of the assay for detection of infectious prion material. This part of the project was hampered by the late availability of suitable containment facilities, which impacted on the number of assays possible using infectious material, therefore full evaluation of the use of the developed methodology for this purpose was not possible. An ELISA was set up using infectious MBH (iMBH) 6.2.7 and the results are

shown as a graph in Figure 6-6. The results show detection of 0.32 $\mu\text{g.mL}^{-1}$ of non-proteinase K digested iMBH within this format.

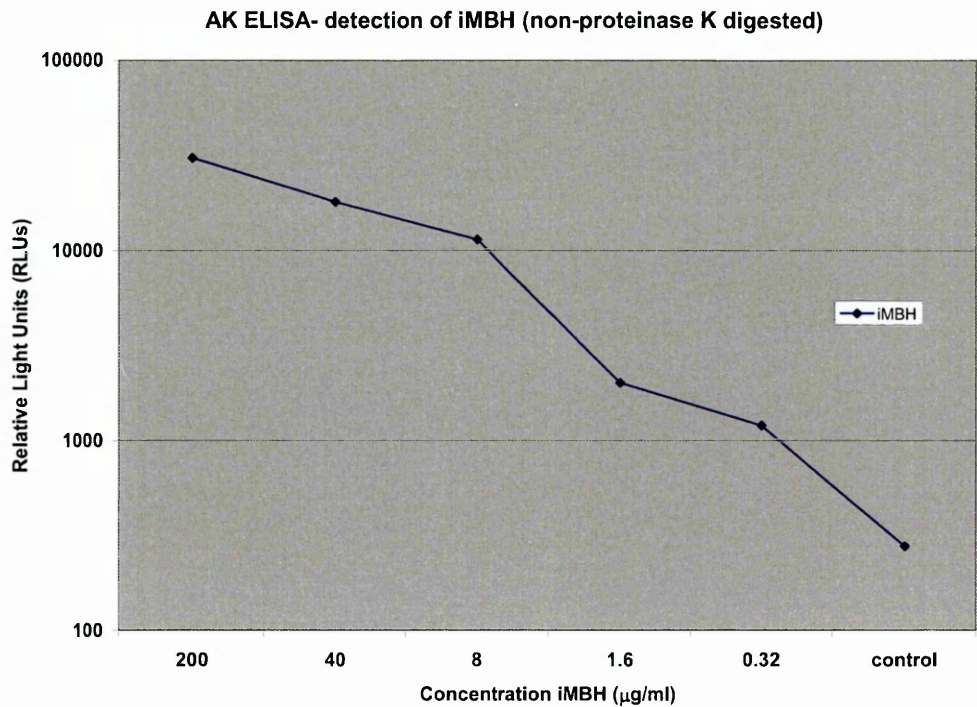


Figure 6-6 Detection of non-proteinase K digested iMBH by AK-ELISA

The iMBH was diluted directly on the plate and detected using a standard AK ELISA. The detection limit is calculated as negative control + 3 x standard deviation; cut –off calculated as 544)

6.3.8 Detection of iMBH on the surface of surgical steel disks

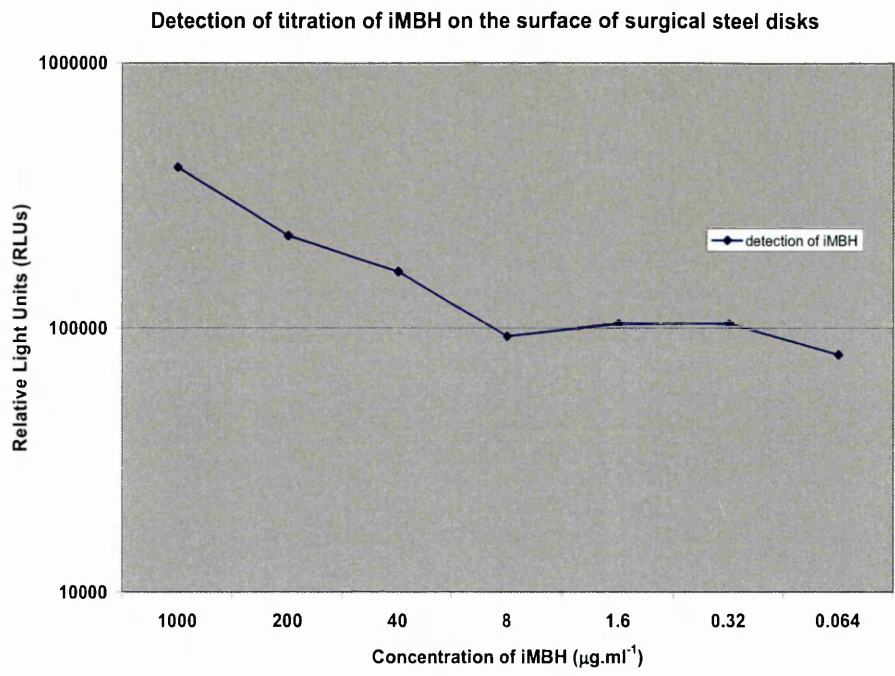


Figure 6-7 Detection of iMBH on surface of surgical steel disks

iMBH was diluted from a starting concentration of $1000 \mu\text{g.mL}^{-1}$ in 5-fold dilutions across a microtitre plate containing 5 mm surgical grade stainless steel disks and allowed to dry. After washing the AK-ELISA was carried out as standard. The results indicate that there is some detection of iMBH on the surface as shown by dose response curve and detection limit of $\sim 40 \mu\text{g. mL}^{-1}$ achieved.

The studies were extended to look at direct detection of prion material on stainless steel disks. Figure 6-7 shows the results obtained in standard ELISA format with iMBH diluted and coated directly onto stainless steel disks mounted within a microtitre plate. Significant issues were observed with non-specific binding of the 6H4-AK conjugate to the control (“unblocked” stainless steel), which made the results difficult to interpret. It was not possible to identify specific binding to iMBH components below the $40 \mu\text{g.mL}^{-1}$ ($4 \mu\text{g}$) shown.

A number of methods were investigated in order to overcome the problems of non-specific binding of the AK to the wells in the stainless steel disk assay e.g. methods of pre-coating the microtitre wells with non-binding material before the disks were glued on however this was not resolvable. On further investigation it was apparent that the observed background binding to surgical steel was due to the thermostable AK component of the conjugate which appears to binds very tightly to steel. The potential to use this property to model protein adsorption and desorption, relevant to prion-directed cleaning and decontamination processes, is being now being explored on separate projects taking place at HPA-Porton Down.

6.3.9 Comparison of AK-ELISA with HRP format

In order to assess the potential benefits of the developed AK ELISA particularly in terms of sensitivity and ability to detect antigen within an excess of biological material, a comparison was made with detection of rec PrP using a traditional HRP format. The results of detection of rec PrP alone by both the AK-ELISA and with HRP assay is displayed alongside the results of detection of rec PrP within a complex biological background. The HRP assay was performed as described (6.2.9) and these results compared direct detection of rec PrP within a microtitre plate with detection of rec PrP within neuronal tissue and sera. Sensitive detection of PrP by the AK-ELISA is demonstrated with limits of 15 pg.mL⁻¹ (equivalent of detection of 15 pg) directly and in the presence of neuronal tissue and blood. The results of this comparison demonstrate that the AK-ELISA format using 6H4 is approximately 100-1000-fold more sensitive than the HRP assay format.

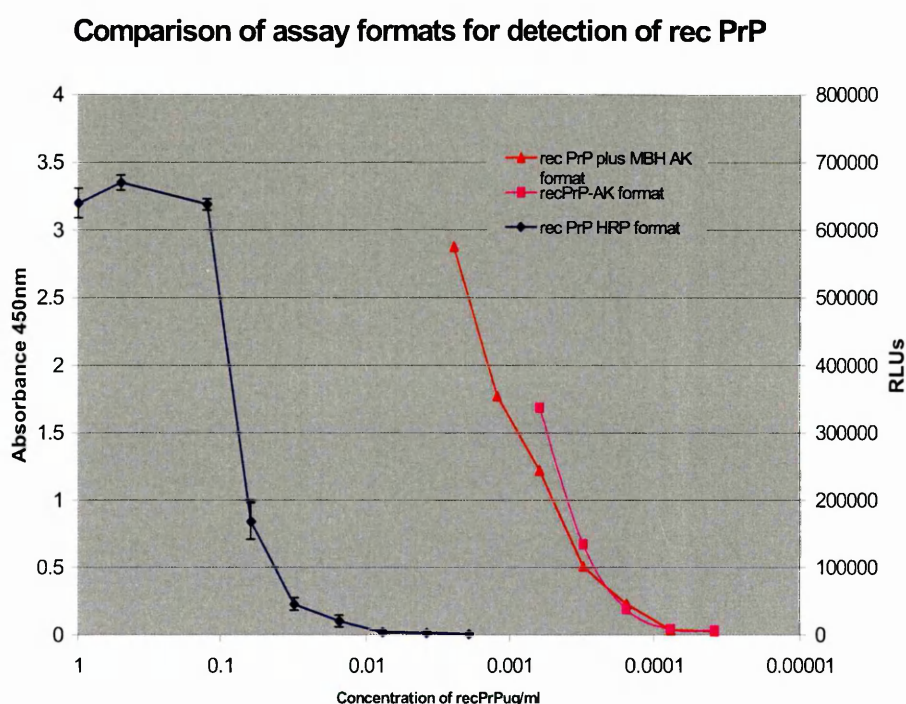


Figure 6-8 Comparison of assay formats

Detection of rec PrP using 6H4 and an HRP assay was compared to detection using the 6H4 alone and within a 50,000 fold excess of MBH and demonstrated that detection using the novel AK –ELISA is 100-1000 fold more sensitive than traditional HRP-ELISA.

The limits of detection achieved by the AK-ELISA for rec PrP alone, on surgical grade stainless steel and within biologically relevant substrates such as neuronal tissue, blood and sera, are summarised in Table 6-1.

Figure	Sample	Detection limit (ng.mL ⁻¹)
Figure 6-1	Rec PrP (in buffer)	0.015
Figure 6-2	Rec PrP on stainless steel	40
Figure 6-3	Rec PrP within MBH	3.91
Figure 6-4	Rec PrP in whole blood	1.6
Figure 6-5	Rec PrP in serum	1.6
Figure 6-6	iMBH in buffer	320
Figure 6-7	iMBH on stainless steel	4000

Table 6-1 Summary of detection limits achieved by AK ELISA

6.4 Discussion

The ultimate goal was to develop an assay that could be routinely adopted within a hospital setting. Therefore, the developed ultra-sensitive assay format required the design of a number of unusual features to make it applicable to the specific problems associated with detection of prion proteins and specifically for the use for validation of cleaning and decontamination protocols. Thus the assay was required to be ultra sensitive and able to detect PrP in complex mixtures and finally to allow practical separation of the binding / washing and assay phases to enable use for detection on the surface of surgical instruments. A further aim was to assess the practicality of the design and consider implications for use in clinical settings and possible conversion to a high throughput format.

In experiments reported in Chapter 4, AK-conjugates prepared with the commercial antibody 6H4 showed significantly better detection of rec PrP than those produced using in-house polyclonal antisera. Therefore, to allow comparison of the assay results with other published material given the relatively poor recognition of rec PrP by the polyclonal antibodies and poor availability and performance of other anti-prion monoclonal antibodies, the 6H4 conjugate was used for further development. Therefore the focus of this phase of the research was to use the results reported and discussed in the previous chapters to enable development of a flexible ultra-sensitive assay format described, for evaluation for detection of prion protein alone, on the surface of surgical steel and in the presence of biological complex material such as tissue, blood or sera.

A working stock of 6H4-AK conjugate was prepared to enable development and optimisation of all stages of the AK-ELISA for detection of prion protein. A number of parameters of the assay methodology were optimised at this stage using detection of a defined concentration of rec PrP with 6H4-AK. Details of all the parameters examined and ideal conditions identified are described in sections 6.2.2 and 6.3.1 and included identification of the following conditions; conjugate dilution and incubation conditions; blocking of non-specific binding; optimal cleavage of AK moiety from bound conjugate; incubation of ADP substrate for maximum ATP production; temperature and duration of heat inactivation steps; in addition to a number of technical features of this assay regarding the type of microtitre plate, heat inactivation method etc.

After the initial optimisation the bioluminescent prion AK ELISA was evaluated to determine the limits of detection of rec PrP alone. The results showed that the use of this assay had enabled detection of 15.6 pg.mL^{-1} of rec PrP, thereby demonstrating the potential of this format to achieve high levels of sensitivity. The results were shown to be statistically significant and the repeated assays carried out during the optimisation phases were used to determine a reliable cut-off value defined as: negative control + 3 x standard deviation, for use in subsequent assay development.

It was clear that the assay was suitable for direct detection of antigen on the surface of a microtitre well; therefore the next stage was to evaluate the use of the prion AK-ELISA for detection of rec PrP on an alternative surface of surgical grade stainless steel. The use of surgical grade stainless steel coupons was used as a model for the surface of surgical instruments. This assay was technically difficult and quite problematic due to difficulties in affixing the stainless steel disks to the base of a 96 well microtitre plate and subsequent problems associated with the disks being dislodged from this surface during the washing procedure. In order to set up ELISA type assays there is a range of commercially available

microtitre plates for use with a range of well defined protein binding characteristics which can be suited to the purpose. It was difficult however to establish the binding conditions for proteins to surgical steel and it was obvious during the course of establishing a limit of detection for this assay, that there was a problem with consistently high background counts even within the control wells, which was not solved by using any available blocking methods. Evaluation with AK alone, established that there was some suggestion that the AK was binding to the stainless steel and thereby causing a high background signal. However, despite this high background, a dose response of rec PrP was detectable using this assay even on the surface of surgical steel and showed detection of approximately 40 ng.mL⁻¹. These results demonstrated that although there were clearly problems with some non-specific binding that there was a potential for further development for detection of prion material on surgical instruments for the validation of cleaning and decontamination protocols. The assay also demonstrated the use of the flexible format allowing the binding of the antibody-AK conjugate to be separated from the detection to enable direct detection on the surface of surgical instruments using this method. It was also hypothesised that the properties of the infectious agent itself would aid binding to stainless steel mimicking the situation seen with surgical instruments and it was therefore anticipated that when the assay was repeated using infectious brain material that the results would be improved.

A further requirement of the assay was the ability to detect PrP in the presence of complex biological samples. One of the samples examined as being relevant to likely end use for the assay was the detection of rec PrP in the presence of non-infectious mouse brain homogenate (MBH) and the results demonstrated sensitive detection of rec PrP within a vast excess of brain tissue. The use of the thermostability of the AK reporter label enabled detection to take place without additional steps and without compromise to sensitivity. The limits of detection achieved by the assay was well above the control value and suggested

that there was the potential for increased sensitivity of detection even within this type of biological background and present format without additional optimisation.

Other examples of biological relevant substrates for detection which would be relevant to the end use are blood and sera particularly as it has been identified that there is still an urgent requirement for the development of blood for screening assay and also as a diagnostic test (Grassi *et al.*, 2008; Ironside, 2006). In our study, experiments were carried out using whole blood and sera as diluents for dilutions of rec PrP and again it was demonstrated that very sensitive detection was achievable of 160 pg, showing that sensitive detection was not adversely affected by the presence of blood. At the lowest concentration level detected the results were still significantly above that of the control demonstrating that potential increases in sensitivity were possible within the existing format. In addition the results of detection of rec PrP in the presence of sheep's sera also demonstrated the same detection limits. The levels of infectivity present in blood has currently been estimated as in the range of 0.1-10 pg.mL⁻¹ (Brown, 2001; Cervenakova *et al.*, 2003). These results therefore opened up the possibility with some further optimisation that this assay may be suitable for detection of prion material in blood products particularly with detection limits achieved using rec PrP alone shown to be 15.6 pg.mL⁻¹. It is therefore approaching the levels of sensitivity desired for this use with perhaps some additional optimisation to increase the levels of sensitivity of detection. However a certain amount of caution is required before extrapolating results obtained using recombinant version of the normal prion protein PrP^C and indeed spiked infectious brain material to detection of prion infectivity in blood. The actual form of the infective prions in blood is presently unknown however, the suggestion that its conformation may be different in circulating blood and in brain tissue suggests that the problems may not only be in terms of sensitivity (Brown 2001).

The assay was evaluated to detect rec PrP on surgical steel surfaces and in complex biological samples as analogues to the samples that would be examined in a clinical setting. The use of the assay to specifically detect infectious material was hampered by lack of suitable containment facilities to enable this work to be completed until very late within the duration of the project.

Using the standard mouse passaged BSE-301V infectious mouse brain homogenate (iMBH) detection of PrP without proteinase K digestion was assessed and the results shown in Figure 6-6 showed detection limits for iMBH as 320 ng.mL^{-1} . Due to the lack of a proteinase K step prior to detection, it was difficult to equate the detection limit achieved with levels of actual infectivity. However it had been previously noted that the relative proportion of disease-associated prion based on relative signal of MBH and iMBH at the same protein concentration demonstrated that iMBH consistently showed a 10-fold higher signal to MBH (results not shown) which suggested that around 90% of the signal was due to the disease associated form, (using the assumption that MBH contains only PrP^{C} whereas iMBH contains PrP^{Sc} and PrP^{C}). Extrapolating from the original protein concentration of iMBH used which was 200 mg.mL^{-1} (10% homogenate $\approx 10^8$ infectious units) the detection limit achieved was estimated as ~ 16 infectious units per well. This therefore demonstrates the potential sensitivity of the detection of prion infectivity using the AK-ELISA within this microtitre plate format.

A further attempt was made to detect iMBH on the surface of surgical steel disks and it was anticipated that the use of infectious material would bind to this type of surface more effectively than non-infectious recombinant prion protein due to the binding characteristics of the altered form of the infectious protein. However detection was hampered by non-specific binding of the AK label to the surgical steel disks. This was rather unexpected and it has been postulated that it is due to the condensed hydrophobic nature of the AK enzyme

(Vieille & Zeikus, 2001; Vonnheim *et al.*, 1998). This specific property of *S. acidocaldarius* AK, although not useful for the identified purpose of this assay, is now being explored for use as a potential indicator method relevant to decontamination and sterilisation processes relevant to infective prion material.

Another measure of the increase in sensitivity of detection of rec PrP was in straight forward comparison to the use of 6H4 antibody using a traditional HRP format. Further optimisation of the AK-ELISA had taken place and which included the use of injectors to deliver the luciferin–luciferase reagent to improve the speed and maximise the RLU values obtained, combined with the use of a heat block instead of a thermocycler for the heating steps and microtitre plates with increased binding capacity. This fully optimised assay was used for comparison with detection of rec PrP using 6H4 and HRP ELISA. The results showed that detection using the novel assay was approximately 100-1000 fold more sensitive than traditional HRP-based ELISA. In addition, further optimisation of the ultra-sensitive assay had increased detection of rec PrP to 400 fM rec PrP both directly and even in complex biological samples such as neuronal tissue and blood

The results therefore showed the considerable potential of this novel ultra-sensitive detection assay system and signified that the AK-ELISA format was suitable particularly to be applied for the detection of prion material in blood or other clinical samples. In order for the AK-ELISA format to be useful for validation of surgical instrument decontamination, however there would need to be a substitution of the AK used as the reporter enzyme to an alternative AK that showed less adherent properties to stainless steel.

7 Discussion

7.1 vCJD - a public health issue?

The possibility of surface contamination of surgical instruments with CJD infectious material remains a significant public health issue raising the possibility of onward transmission. The total number of deaths from definite or probable vCJD is 163 to date (March, 2008) with a further 3 cases incubating disease (The National Creutzfeldt-Jakob Disease Surveillance Unit (NCJDSU) 2008) which in terms of numbers affected is relatively low. This has given rise to speculation that the vCJD “epidemic” is coming to an end and therefore no longer poses a risk to public health. However, given the widespread exposure of the UK population to BSE in the 1980s and early 1990s, it is possible that there are significant numbers of people incubating the disease. This possibility has been strengthened by the publication of a prevalence study, (Hilton *et al.*, 2004a) which retrospectively examined appendix and tonsil pathology samples. The results indicated that numbers may be significantly higher than the current known clinical cases. Furthermore, whilst all the clinical cases of vCJD have been homozygous for methionine at codon 129, there is now evidence that both valine homozygous (Hilton *et al.*, 2004a; reviewed by Ironside *et al.*, 2006), and heterozygous genotypes (Peden *et al.*, 2004) may also be capable of acquiring the disease. These cases are currently sub clinical in presentation, however the clinical outcome is unknown and there is a great deal of uncertainty surrounding the actual levels of risk. Studies on the human prion disease Kuru (Collinge *et al.*, 2006) and also from transgenic animal studies (Bishop *et al.*, 2006) have raised the possibility that very long incubation periods perhaps even extending beyond a normal average life span may be possible. These studies, taken in context with the results of the prevalence study could raise the possibility of the presence of sub clinical infections within

the UK population. This is particularly true for persons not genetically pre-disposed to develop disease by being either valine homozygous or heterozygous at codon 129. Whether these people represent a further risk to the more susceptible individuals via surgery, transplant or blood transfusion remains unknown. Cases of iatrogenic transmission of inherited and sporadic CJD by surgery have been reported in the scientific literature (Brown *et al.*, 2000; Will & Matthews, 1982). Although there are no known cases of iatrogenic transmission of vCJD by surgery there is convincing evidence of vCJD transmission by blood transfusion (Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006; HPA 2007). In addition, the tissue distribution of vCJD has been shown to be markedly different to that of other human forms of CJD with identification of infectivity associated with the lymphoreticular system, including tonsil, spleen and lymph nodes which may mean that that iatrogenic transmission of vCJD may be a greater threat (Hilton *et al.*, 2004b). In addition, a number of recently published studies have highlighted that current decontamination practices within sterile service departments (SSDs) may require general improvement as significant levels of protein surface contamination was identified on surgical instruments post processing and at point of use (Baxter *et al.*, 2006; Lipscomb *et al.*, 2006a; Lipscomb *et al.*, 2006b; Murdoch *et al.*, 2006). It is clear therefore that there is potential for transmission of CJD through a number of routes such as surgery and blood transfusion and as such there remains a risk to public health until improved cleaning and sterilisation techniques are in place suitable for inactivation of prion infectivity with practical methods of validating the process and development of a sensitive detection assay suitable for screening of blood (Ironside, 2006).

7.2 Aim of research

This research project aimed to address the problems associated with sensitive detection of prion proteins by the development of a novel ultra-sensitive detection method based on a thermostable adenylate kinase (AK) reporter antibody label. To achieve these goals the assay needed to take into consideration the current understanding of the nature of prions in blood and the very clear gaps in our knowledge (particularly related to the form of prions in blood and the observed adherence of prions to steel surfaces), whilst maintaining a clear view of how such an assay would be applied by different end-users. This study describes the development of this methodology including investigation of potential for ATP generation by the enzyme to be coupled to bioluminescent detection. The developed methodology has the flexibility to enable direct detection on surgical instruments and within biological substrates such as blood and tissue with utilisation of the thermostability of the AK. It would also be very easy to alter the method to incorporate new reagents as the understanding of the nature of prions develops in the future.

7.3 Development of a model assay

Significant problems have been encountered in attempts by researchers to develop assays for sensitive detection of CJD and it has been hampered by lack of specific antibodies for the disease associated form of the prion protein. It has been acknowledged that the prion protein is poorly immunogenic (Prusiner *et al.*, 1993) and due to the nature of the infectious agent being an altered form of a normal protein, there are few antibodies produced which claim to preferentially recognise the disease associated form of the protein (Korth *et al.*, 1997; Moroncini *et al.*, 2004). Given the number of decontamination methodologies currently being developed (Baxter *et al.*, 2005; Fichet *et al.*, 2007; Heindl *et*

al., (in press); Jackson *et al.*, 2005; McLeod *et al.*, 2004; Whittaker *et al.*, 2004) there is clearly an urgent requirement for validation of these processes other than by bioassay. It was decided that due to the lack of available sensitive and specific antibodies, that the priority was to focus on the detection end of the assay to enhance the overall sensitivity as far as possible.

The ultimate goal was to develop an assay that could be routinely adopted within a hospital setting and therefore it had to be relatively cost effective and applicable to standard hospital laboratories. Adenylate kinases (AKs) have been previously discussed as potential enzymatic reporters for conjugation to antibodies for immunoassay use (Gadow *et al.*, 1984). It was identified that the use of AKs for this purpose would offer a number of advantages over conventional alkaline phosphatase or horseradish peroxidase conjugates due to the significant amplification of signal via generation of ATP, coupled to sensitive bioluminescence detection. This study looked at the use of mesophilic AKs for this purpose however had failed to generate enzymatically active antibody conjugates. For the purpose of this study therefore, it was decided to look at the possibility of using an alternative source of AKs isolated from thermophiles and hyperthermophiles for conjugation to antibodies, in the anticipation that the more inherently robust nature of these enzymes would overcome the problem of loss of enzyme activity on conjugation (Vieille & Zeikus 2001). Another advantage was that potential use of the inherent thermostability properties of the enzymes to enable inclusion of thermal denaturation steps within the assay to effectively remove background signal resulting from AK present in contaminating tissue and therefore enable direct detection without requirement for pre-processing of the sample.

The use of ATP bioluminescence has been described previously as a sensitive methodology (reviewed by Kricka, 1995; Squirrell & Murphy, 1997). It also had the

advantage that reagents and instrumentation are commercially available and in formats suitable for high throughput laboratory use or more portable applications. Many of these would support rapid read-out of results, perhaps suitable for use within sterile service departments to validate decontamination processes. The use of ATP bioluminescence has been extensively applied for hygiene monitoring (Stanley, 1989) and subsequently further refined to utilise the amplification step available by the use of recycling of ATP by AK and to enable specific detection of bacteria by release of internal AK from cells by bacterial phages (Blasco *et al.*, 1998; Squirrell, Price & Murphy, 2002).

The use of a thermostable AK as an enzyme reporter has not been described in the scientific literature; however there was clear potential for sensitive detection using the proposed assay format. The development of a model assay was important because it demonstrated the feasibility of the assay particularly that successful conjugation of an antibody to a thermostable AK was possible, without loss of biological activity of the enzyme. It also enabled establishment of other assay parameters to overcome some of the practical difficulties of detection taking place on the surface of surgical instruments. This necessitated the development of a method to separate initial detection with generation of signal and therefore a method of cleavage of the AK from the bound antibody was developed. In addition establishment of whether it was possible to create conditions required to generate ATP from the enzyme reporter within this novel use of AK.

Therefore development of the model assay, using *B. stearothermophilus* AK was successful in establishing 'proof of principle'. It also highlighted that although the parameters of the assay were established in principle that in order for the proposed method to be used for the purpose of detection of prion protein that a number of changes to the format would be required. Particularly sourcing of a more thermostable AK, identification

of a suitable anti-prion antibody, alongside optimisation of the methodology to maximise sensitivity.

7.4 Selection of improved thermostable adenylate kinases

The development of a model system demonstrated that the proposed assay methodology was possible and therefore the focus became to adapt the system to enable sensitive detection of infective prion material. One of the main achievements within development of the model assay was the successful conjugation of *Bacillus stearothermophilus* AK without loss of the enzyme activity, seen previously using mesophilic AK (Gadow *et al.*, 1984). It had been identified however, that the continued use of this enzyme for further development of the assay was not desirable, as results of heat stability studies showed that there was considerable loss of activity on heat steps up to 70 °C. The use of thermostable properties of the enzyme was crucial to enable direct detection of PrP within a potential excess of biological material as found on surgical instruments. This is due to the presence of thermolabile AK which is ubiquitous throughout cell types and therefore inclusion of sufficient heat steps would be a requirement of the assay. The next stage was to further progress the assay by identification of an alternative AK with superior qualities of thermostability and which could be produced recombinantly in order to enable production of sufficient quantities to enable development of the assay.

This was accomplished using a couple of different approaches, as the availability of a thermophile culture collection (CEPR Thermophile Culture Collection) meant that a number of thermophilic microorganisms could be screened for potential candidate AKs and in addition a source of recombinant *Sulfolobus acidocaldarius* AK in the form of a plasmid DNA was kindly donated by Professor G Schaeffer (Vonnheim *et al.*, 1998). This

was successfully transformed and over-expressed in *E. coli* and subsequently purified and provided an initial stock of AK for use in the assay development. However, it was identified that there was a low yield upon over-expression of 0.5 mg per Litre of culture meant that this was not ideal for continued use and it was also hoped that AKs with more desirable properties would be identified from the screening process. The results of the screening procedure identified some potential candidates with the desired thermostability and two of these were successfully produced recombinantly, which were *Sulfolobus acidocaldarius* AK and *Sulfolobus solfataricus* AK and subsequently purified AK was conjugated successfully to anti-prion antibodies as described in Chapter 6.

The reason why thermostable AKs were able to be conjugated to antibody without inactivation compared to mesophilic AK is not entirely clear. It is known that enzymes isolated from thermophilic and hyperthermophilic microorganisms tend to be more compact and denaturation resistant than mesophilic enzymes (Vieille & Zeikus, 2001). The extreme thermostability exhibited by some AKs has resulted in studies which have aimed to elucidate the structural and biochemical properties which confer this property (Bae, Phillips, 2004; Bae & Phillips, 2006; Vieille & Zeikus, 2001). One of the main factors is thought to be maintenance of flexibility of the protein molecule at the required environmental temperatures and this is influenced by intramolecular interactions. However even within classes of AKs that have the ability to survive at extreme temperatures there is considerable variation. The biochemical composition and structure of AKs isolated from the extremophiles *S. acidocaldarius* and *S. solfataricus* have been shown to be trimeric in structure with an internal hydrophobic core and it was subsequently identified that they belong to a novel class of AKs (Kath, Schmid & Schafer, 1993; Okajima *et al.*, 2002; Vonnrhein *et al.*, 1998). It has been hypothesised that the trimeric nature of the structure could be responsible for conferring the thermostability of the protein (Vonnrhein *et al.*, 1998). It is tempting to postulate that it is the robustness of the protein structure as a result

of the trimeric conformation that resulted in successful conjugation to antibodies without loss of biological activity. However on analysis of structural composition of *B. stearrowthermophilus* AK which was also conjugated successfully to antibody has revealed that this AK is monomeric in structure and although it is clear that this AK is less thermostable than *S. acidocaldarius* AK, conjugation to antibody was achieved without loss of enzyme activity (Berry, Phillips, 1998; Glaser *et al.*, 1992). Additional analysis of the structure of *B. stearrowthermophilus* AK has identified that it has a structural zinc atom unlike that of the AK from the mesophile *Bacillus subtilis* with which it has a high sequence identity (Glaser *et al.*, 1992) and it has been hypothesised that it is the presence of this zinc atom that confers the thermostability. This has been further substantiated by a study of an AK from a different thermophile *Thermotoga maritima* which also contains a structural zinc, which demonstrates some suggestion that its removal results in loss of thermostability (Vieille *et al.*, 2003). This is interesting, however since not all thermophilic AKs have this structural zinc atom; it is obviously not the only explanation in terms of conferring thermostability. Further investigation suggests that the balance between stability and flexibility is crucial for proteins to operate at their environmental temperatures and the differences are at the level of intramolecular interaction level (Bae & Phillips, 2004). Therefore there does not appear to be one common or distinct feature responsible for conferring thermostability or clarifying the reasons that enabled successful conjugation to take place for the AKs used within this study, without loss of activity in comparison mesophilic AKs.

The production of recombinant AKs was hampered by the comparative scarcity of known genomic sequence for the thermophiles, however a few were available including *Pyrococcus abyssi*, *Thermatoga maritima*, *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*. Evaluation of over-expression in *E. coli* and heat stability studies revealed recombinant AKs produced from *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*

AKs were the most thermostable and active enzymes and therefore work concentrated on these AKs for the purpose of production and purification of sufficient quantities for development of the assay. The thermostable nature of the proteins was able to be utilised within the purification process and established as a very efficient first stage of purification for removing a large proportion of the thermolabile proteins resulting from *E. coli* host strain and achieving approximately 80% purity before affinity purification.

One rather surprising result from the characterisation of *S. acidocaldarius* AK was that it was as active at 37 °C as at 80 °C. This has been described previously for AK isolated from the hyperthermophile *Thermotoga neapolitana* (Vieille *et al.*, 2003). This unusual ability demonstrated further the suitability of the use of this enzyme for use in the development of an ultra-sensitive assay format as the enzyme was clearly robust and unlikely to be affected by all the various assay conditions encountered with a potential to reach very sensitive detection limits. Furthermore the enzyme activity was shown to be linear over greater than 8 logs and has shown considerable potential for sensitive detection to less than 100 attomoles.

7.5 Production of anti-prion polyclonal antisera

One of the key components for the development of an ultra-sensitive prion ELISA was the availability of anti-prion antibodies capable of recognising the putative disease associated form of PrP (PrP^{Sc}), however this remains a significant issue for the development of immunoassays (Gilch *et al.*, 2003). A decision was taken that in house polyclonal antibodies would be produced based on a novel approach. The approach which was adopted within this study was to attempt to increase the antibody response to prion mimetic peptides by combining a method using cyclic peptides to potentially mimic native

conformational structure of the proteins (van der Werf *et al.*, 1994) with the use of an alternative carrier protein using purified protein derivative (PPD) in conjunction with pre-immunisation with BCG which has been previously shown to boost the serum response (De Silva, Egodage & Wilson, 1999). For the purposes for detection on the surface of surgical instruments, it was not considered necessary to differentiate between the normal form of prion protein (PrP^C) and the protease resistant form (PrP^{Sc}) because the presence of either isoform would indicate that the decontamination process had been inadequate. However for the developed assay to be suitable for other purposes such as a diagnostic or for screening of blood donations then preferential recognition of the putative disease isoform would be desirable.

Characterisation of the anti-prion polyclonal sera produced using these strategies showed reasonably good reactivity to the recombinant version of normal PrP^C in both ELISA and Western blots, however the commercial antibody 6H4 showed significantly better detection of rec PrP and additionally showed detection of the characteristic PrP^{Sc} bands within protease digested iMBH. It was therefore apparent that despite the use of strategies to attempt to increase the success of production of specific anti-prion polyclonal antibodies that the antisera produced were of limited use for the further development of the assay.

It was decided therefore that work would focus on the use of the commercially available monoclonal antibody 6H4, with the advantage that it would enable comparison of the assay results with other published material (Barletta *et al.*, 2005; Biffiger *et al.*, 2002; Lourenco *et al.*, 2006).

7.6 Development of an ultra-sensitive prion ELISA

7.6.1 Detection on the surface of surgical instruments

During the timescale of this project, there was significant progress in optimisation of this assay format for rec PrP and to some extent infectious MBH (iMBH) with some limits of detection established on the surface of surgical steel disks as a model for surgical instruments.

Surgical grade stainless steel coupons were used as model for surgical instruments for evaluation of detection directly on this type of surface. A number of unexpected problems were encountered specifically for detection on stainless steel disks with both the rec PrP and iMBH. When the assay was evaluated for detection of rec PrP on the surface of surgical steel coupons, the results were not as sensitive as those using a microtitre plate format and it was thought that this may be due to the surface of the steel not being designed to support binding of proteins. It was anticipated at this stage that this may be solved in part when detecting infectious material as the altered protein form of the infectious prion are known to adhere to stainless steel more readily. However on evaluation of the results with iMBH on the surface of surgical steel disks, it was found that there were similar problems as seen previously and there was evidently a high degree background signal that was not solved by the methods investigated. One hypothesis was that the relatively poor results combined with the high background signal may be due in part to the extremely tight adherence of the disease-associated prion material to stainless steel surfaces and the lack of availability of suitable epitopes for antibody binding. However, it was clear also that the results were also hampered by binding of the *S. acidocaldarius* AK to stainless steel surfaces in their own right, giving rise to relatively high background

signal. This property of the thermostable *S. acidocaldarius* AK to bind tightly to stainless steel was clearly a disadvantage for the purposes of development of an assay for detection of prion infectivity on the surface of surgical grade steel. However, it may have potential use as an indicator for decontamination or cleaning processes relevant to prion inactivation.

The properties of adherence to surfaces like stainless steel was strangely reminiscent of the prion infectious agent itself and may have something to do with the structure of the *S. acidocaldarius* AK with its trimeric structure with central β -sheet region and highly condensed hydrophobic nature (Vonnheim *et al.*, 1998). The AKs from *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* which have also been shown to be trimeric structure may represent a separate class of AKs (Kath, Schmid & Schafer, 1993; Okajima *et al.*, 2002). This raised the possibility that alternative thermostable AKs which differ in structure and are perhaps monomeric may have the desired thermostability and activity for use in the assay but without the characteristic adherence to surfaces such as stainless steel. Related immunoassay projects have subsequently made some progress on reducing the background binding to surfaces by using alternative thermostable AKs (results not shown) however this was after the completion of this project.

7.6.2 Detection of rec PrP

Results of detection of prion infective material on the surface of surgical steel coupons, was rather disappointing as although detection of rec PrP and iMBH was achieved, the sensitivity was rather low and the detection limits were not as sensitive as seen when using the traditional microtitre plate format.

It was clear however that the developed AK-ELISA within a traditional microtitre plate format was capable of very sensitive levels of detection comparable with most current published studies. With detection limits optimised to approximately 0.6 pM (15 pg.mL⁻¹) of rec PrP the assay compares favourably with other published immunoassays for PrP. (Grathwohl *et al.*, 1997) report that detection of scrapie infected murine brain material by ELISA was as sensitive as Western blotting with detection limits in the region of 5- 20 µg.mL⁻¹; however additional steps were required to effectively remove the contaminating background biological material by detergent and protease digestion prior to detection. Another study (Yamamoto *et al.*; in press) reports detection limits for rec PrP of 20-65 µg.mL⁻¹ and again this assay involved a protease and detergent pre-step before detection. In a more direct comparison, (Biffiger *et al.*, 2002) shows a detection limit of 30 pg.mL⁻¹ PrP^{Sc} using the antibody 6H4 in a luminescence-based assay. The use of a method of immunocapillary electrophoresis has also been evaluated for detection of abnormal prion protein in blood samples of CJD patients. (Lourenco *et al.*, 2006) however the results demonstrated a relatively low sensitivity of detection of rec PrP within the nM range. Another published study (Barletta *et al.*, 2005) describes an unusual and rather nice approach which utilises a PCR reaction to amplify the signal subsequent to standard ELISA detection termed immuno-polymerase chain reaction (IPCR). Very sensitive detection has been shown using this method with detection of 1 fg.mL⁻¹ of rec PrP and detection of 10-100 infectious units within scrapie infected hamster brains. The researchers within this study have used a similar approach to that reported within this study in that the focus is to improve the detection end of the assay because of a lack of disease specific antibodies. This is clearly a novel approach with a great deal of potential, however it is perhaps too complex to be applied to general clinical use and there is an additional reported problem of false positives which it is thought to be due to inadequate proteinase K digestion prior to detection.

As with any antibody based assay system, the detection limits achieved are dependant on the quality of the antibody used. The commercially available anti-prion antibodies, including 6H4 are relatively poor compared to antibodies developed for detection of other diseases which tend to have a better specificities and sensitivities. This has therefore been a limiting factor on the ultimate sensitivity of the developed assay and availability of an improved antibody such as a higher affinity disease specific monoclonal antibody would be desirable and would provide a boost to the sensitivity.

One example would be the use of the conformation dependant specific antibody described in published studies (Safar *et al.*, 2002; Safar *et al.*, 2005). The use of this approach reportedly recognises the protease sensitive as well as the protease resistant forms of disease associated isoform PrP^{Sc}. This may be important given the recent reports of efficient transmission of disease despite the absence of significant levels of PrP^{Sc} immunoreactivity following proteinase K digestion and Western blotting (Barron *et al.*, 2007). However the reported detection limits of infectious prion material using this using this conformation specific immunoassay approach (Safar *et al.*, 2002) is in the region of approximately 1 ng.mg⁻¹ and this raises the possibility that the combination of AK-ELISA detection, with limits in the pg.ml⁻¹ range, and a conformation specific antibody may enable a significant increase in sensitivity.

7.6.3 Detection in blood

During the course and subsequent to this study, three cases of vCJD attributed to blood transfusion have been published (Llewelyn *et al.*, 2004; Peden *et al.*, 2004; Wroe *et al.*, 2006) and a fourth announced via a press release by HPA.

Given the current concern of transmission of vCJD through blood transfusion, it seemed prudent to look at the use of the assay format to see if the required levels of sensitivity could be achieved using the AK ELISA. In order to assess the potential for this purpose, the assay was assessed for detection of rec PrP within whole blood and sera and the results reported in Chapter 6. The current estimate of levels of infectivity in blood used by the National Blood Service and others, is approximately 1-100 infectious units of vCJD prion agent per mL of blood which equates to around 0.1-10 pg.mL⁻¹ prion (reviewed Brown, 2005; Cervenakova *et al.*, 2003). Therefore the current limits of detection for rec PrP of 15 pg.mL⁻¹ is approaching the required sensitivity for prion infectivity in blood and with some further optimisation it may be possible to reach the desired levels of sensitivity. These results were very heartening, however caution is required when interpreting results of an assay using a recombinant PrP and indeed spiked infectious brain material as the actual form that infective prions present in blood is as yet unknown and there is suggestion that the conformation within circulating blood may differ to that within brain material (Brown, 2005).

The detection limit for non-proteinase K digested iMBH using the ultra-sensitive AK-ELISA was calculated as 320 ng.mL⁻¹, which equates to approximately 16 infectious units if it is assumed that the majority of the signal is due to disease-associated PrP. It should be noted, however that these results were obtained on standard microtitre plates and the assay did not perform as effectively on stainless steel surfaces due to problems associated with the currently used *S. acidocaldarius*.

7.6.4 Other applications

The AK ELISA methodology developed here for the detection of PrP is, broadly applicable to other assays where sensitive detection is required in complex biological mixtures. After the end of the period of this study, the use of the AK-ELISA format was explored further for other uses and the results showed exceptional levels of sensitivity. The increases in sensitivity were demonstrated by the substitution of the antibody labelled HRP detection with an AK labelled antibody. The use of this method for detection of rotavirus antigen showed a detection limit of 10 fg.mL^{-1} which was 100-fold more sensitive than the available kit format.

In addition, the use of the AK-ELISA for assessment of improvement of detection of *Clostridium botulinum* type B neurotoxin within an endopeptidase cleavage assay (Hallis, James & Shone, 1996; Shone *et al.*, 1993; Wictome *et al.*, 1999) demonstrated 10,000 fold improvement on the traditional assay (all results Murdoch, Sutton *et al* unpublished). These results show the potential of the developed novel AK-ELISA for ultra-sensitive detection of alternative targets with superior available antibodies.

As the novel assay format was developed during the period of study and to some extent subsequently afterwards, it became apparent that the uses of the assay could be varied to some extent depending on whether it was to be utilised for detection e.g. on the surface of surgical instruments; of antigen within a vast excess of biological material; or for direct ultra-sensitive detection using a straight forward microtitre plate format.

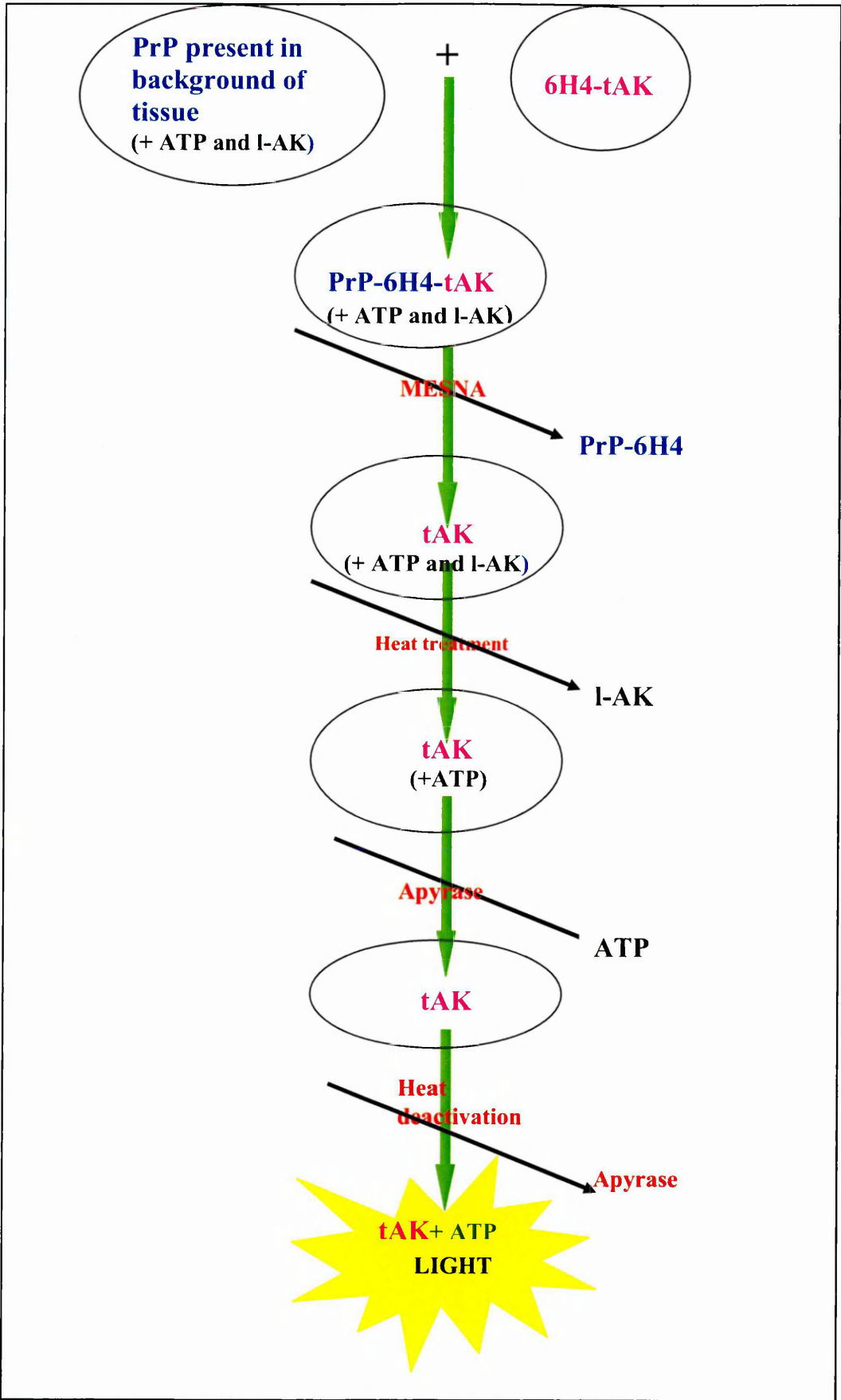


Figure 7-1 Schematic representing the flexible thermostable AK-ELISA format for antigen detection.

7.7 Future work

Due to the fact that the period of study ended some time ago, a few of the desired future work goals have already been explored further. These have included further optimisation of the assay format, which have included modifications in the equipment used to make the process more stream-lined and more applicable to high throughput use.

The results of the research identified that the use of a thermostable AK within this novel immunoassay format was capable of very sensitive detection. This was particularly shown in the case of detection of rec PrP alone and within a background of biological tissue, blood or sera. Indeed the results of detection of rec PrP in blood approached the levels of sensitivity required for use for this purpose. Ideally therefore, future work would focus on the further development of the assay to improve the sensitivity 10-100-fold as indicated by current estimates of levels of prion infectivity in blood. This could be approached in a number of ways. Firstly the substitution of an alternative antibody to 6H4 with disease specific specificity would provide an immediate increase in sensitivity and applicability of the assay (Korth *et al.*, 1997; Paramithiotis *et al.*, 2003; Safar *et al.*, 2005).

The structure of the AKs isolated from thermophiles is of interest to determine and compare their suitability for the purpose within the assay format. This would be desirable to increase understanding and enable easier identification of alternative AKs with superior properties for this purpose. Investigation into the biochemical and protein structure of thermostable AKs has revealed that the reasons for thermostability of AKs is varied and probably multi-factorial. The choice of thermostable AK for use in the assay was limited to some extent by the lack of published sequence data for production of recombinant versions

and more suitable AKs may yet be identified which would further boost the sensitivity of the assay. There have been a number of studies which have shown that protein engineering can successfully be used to promote stability and enhanced activity of enzymes using a technique called directed evolution (Cramer *et al.*, 1998; Giver *et al.*, 1998; Van den Burg *et al.*, 1998; Zhang, Dawes & Stemmer, 1997). This is an *in vitro* technique which mimics natural evolution and has been shown to successfully increase enzyme activity or thermostability. One method involves the use of error prone PCR. The use of this technique was shown to successfully confer thermostability and improve the activity more than 200 fold of an enzyme subtilisin (Zhao & Arnold, 1999). This use of this type of approach would seem to offer real possibilities for the production of a more active thermostable AK and with perhaps the desired properties of lack of surface adhesion.

Therefore in conclusion, the developed assay has demonstrated considerable potential for ultra-sensitive detection of prion protein and other antigens particularly for direct detection of antigens within biological materials such as tissue, blood or sera. There are possibilities, however for further improvement of sensitivity of the assay in terms of improvement of antibodies used and also use of potentially superior AKs either identified by further screening or by the use of protein engineering techniques. The further development of this assay format for the specific detection of prions in blood would ideally be the main focus.

7.8 References

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